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Development of a house dust mite model of mixed allergic airway inflammation and analysis of allergyprotective effects of *Staphylococcus sciuri*

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„You can't connect the dots looking forward. You can only connect them looking backwards, so you have to trust that the dots will somehow connect in your future“

Steve Jobs

Contents

1 Introduction

1.1 The burden of asthma

Asthma is a highly prevalent disease that affects 330 million individuals in the world. During the past 60 years, the incidence and severity of asthma have constantly increased. Great attention has been aroused by scientsists and much effort has been put into investigation in both patients and animal models. It is becoming recognized that allergic asthma is a multifactoral disease of gene-environment interaction (Renz et al., 2011; Mukherjee et al., 2011). Novel phenotypes of asthma such as non-eosinophilic asthma has been identified and current therapy which focuses on the inhibition of Th2 cell-mediated eosinophilic airway inflammation is not sufficient for the treatment of new phenotypes. There is an urgent need to uncover the underlying pathogenesis of different asthma phenotypes, which serves to the direction of appropriate and effective treatment of asthma disease.

1.2 The definition of asthma

What is asthma? The Global Initiative for Asthma (GINA) (Bateman et al., 2008) and the National Heart, Blood and Lung Institute (EPR-3, 2007) describes asthma as follows:

"*Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction within the lung that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli. Reversibility of airflow limitation may be incomplete in some patients with asthma*".

To be brief, asthma has three main features: airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation. Interactions between these features determine the clinical manifestations and severity of asthma (Figure 1.1) and the response to treatment.

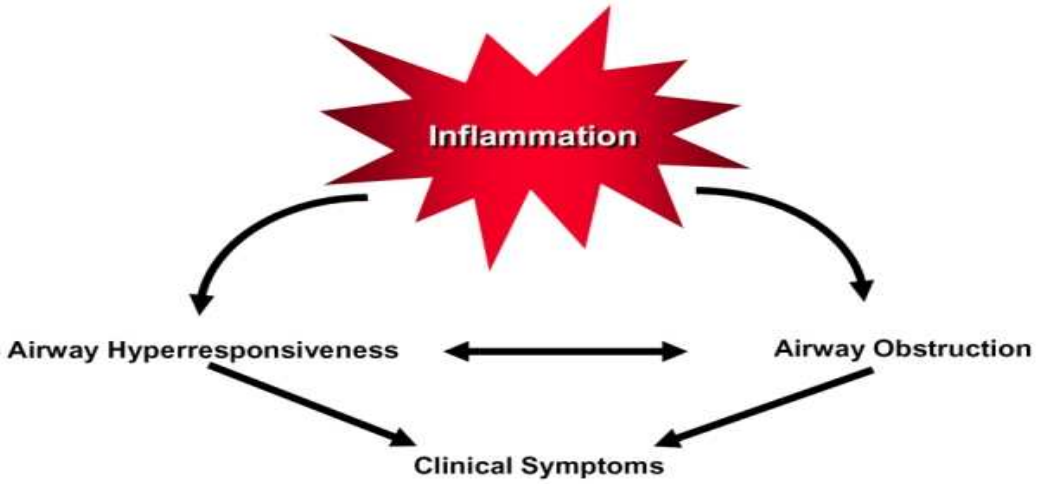


Figure 1.1 Interactions between airway inflammation and the clinical symptoms and pathophysiology of asthma (EPR-3, 2007)

However, the concepts underlying asthma pathogenesis have been renewed tremendously in the past years and are still undergoing updation because various phenotypes of this disease are identified. A better understanding of this disease requires a detailed interpretation of genetic patterns, environmental substances or other factors which are responsible for various clinical manifestations or different subtypes of asthma (Busse et al., 2001).

1.2.1 Asthma phenotyping

Asthma has long been considered as a heterogeneous disease, which consists of different phenotypes. The term phenotype refers to "the visible characteristics of an organism resulting from the interaction between its genetic makeup and the environment" (Wenzel, 2006). Clinically, asthma phenotypes represent the individual differences of disease which are directly related to meaningful outcomes, for example, the response to treatment, rate of progression, prognosis of disease, etc. Therefore it's of

importance to identify a patient's asthma phenotype for the benefit of treatment. Up to now, many categories have been employed to define asthma phenotypes, since asthma is likely to be comprised of overlapping syndromes with multioriginal pathobiology. The categories are mostly based on etiologic factors (eg. aspirin sensitivity, persistent respiratory infections, occupational irritants, or toxic exposures), clinical features (eg. mild, severe, brittle, near fatal, with fixed airflow limitation, steroid resistant), inflammation type (eosinophilic, non-eosinophilic) or gene expression signature (Th2-high, Th2-low) (Wenzel, 2006; Bel, 2004; Woodruff et al., 2009; Bradding et al., 2010). Among all the defined phenotypes, non-eosinophilic asthma is a relatively new phenotype which was first identified by Green's group (Green et al., 2002). In their studies, a distinct subgroup (23% asthma patients) had a sputum cell profile with increased neutrophils but no eosinophils. Such patients with a neutrophil-dominated sputum responded less well to inhaled corticosteroids than those with an eosinophil-dominated sputum. Up to now, a specific management for non-eosinophil asthma phenotype is still lacking. Further phenotyping of asthma provides a vital stimulus for the development of novel therapeutic agents or prevention concepts, thereby improving our insight into the pathogenesis of asthma. A detailed asthma phenotyping is also encouraged by the availability of new therapies.

1.2.2 Pathophysiological mechanisms in the development of airway inflammation

From the definition of asthma, we know that inflammation plays a critical role in the pathophysiology of asthma. As shown in Figure 1.2, airway inflammation involves an interaction of many cell types and multiple mediators within the airways which eventually results in the characteristic pathophysiological features of the disease: bronchial inflammation and airflow limitation that give rise to recurrent episodes of cough, wheeze, and shortness of breath. The processes by which these interactive events occur and lead to clinical asthma are still under investigation. In addition, although distinct phenotypes of asthma exist, airway inflammation remains a consistent pattern. The pattern of airway inflammation in asthma, however, does not necessarily vary depending upon disease severity, persistence, and duration of disease. The cellular

profile and the response of the structural cells in asthma are quite consistent.

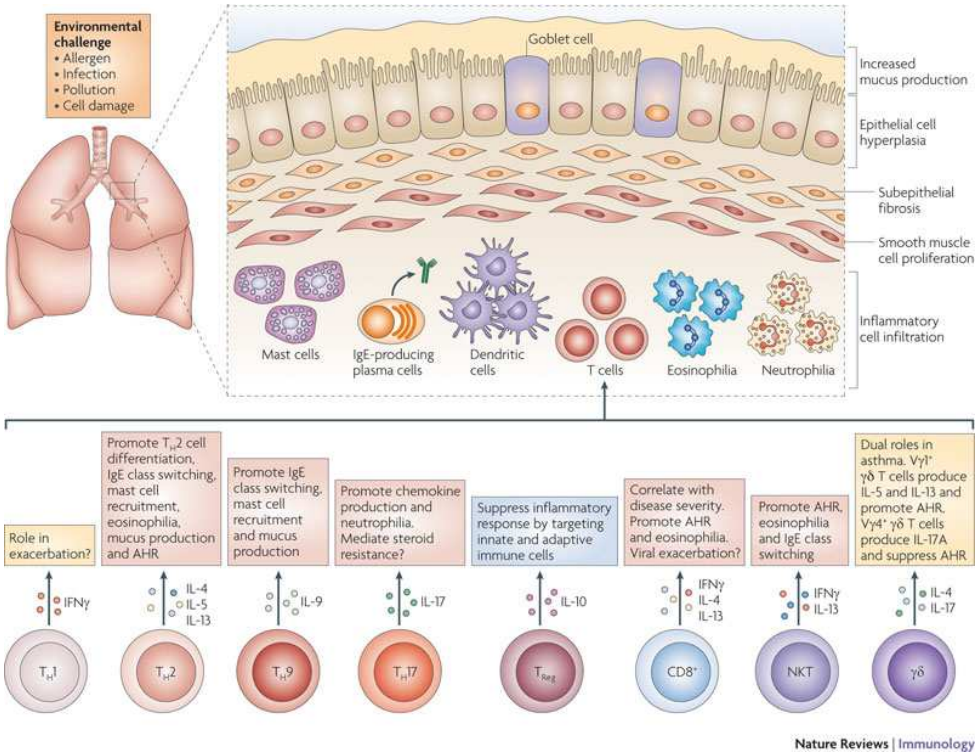


Figure 1.2 Asthma is a multicellular disease of airway inflammation (Lloyd et al., 2010)
Many cells including mast cells, eosinophils, lymphocytes, neutrophils, dendritic cells and epithelial cells are involved in the pathogenesis of asthma.

1.2.3 Mast cells

Mast cells play an essential role in the initiation of allergic (extrinsic) asthma by virtue of their expression of high-affinity IgE receptor, FceRI (Bradding et al., 2006; Andersson et al., 2011). Increased numbers of bronchial mast cells and elevated levels of IgE have been described in allergic asthma, which is triggered by inhaled allergens such as house dust mite, pet dander, pollen proteins, and mold spores (Kearley et al., 2011; Andersson et al., 2011; Goplen et al., 2009; Fairs et al., 2010). When exposed to the allergens, mast cells dergranulate and secrete histamine, prostaglandin D2 and leukotriene C4, cytokines and protease in the airways, which contributes to an early asthmatic reaction (EAR) with bronchoconstriction vasodilatation and mucus production (Woodman et al., 2008; Saha et al., 2009).

1.2.4 Eosinophils

Eosinophils come to rise in the late asthmatic reaction (LAR). They accumulate in the airways of most asthmatic patients to constitute airway inflammation (Williams, 2004). The activated eosinophils release a number of cytotoxic molecules including major basic protein (MBP) and eosinophil peroxidase (EPO), which are responsible for the induction of bronchoconstriction and airway hyperresponsiveness (AHR), and produce cytokines such as IL-5. IL-5 is extremely important for the maturation of eosinophils. It acts on eosinophils to promote terminal differentiation of eosinophil-lineage committed precursors and together with the chemokine eotaxin leads to eosinophil recruitment to sites of inflammation. IL-5 also prolongs eosinophil survival (Kariyawasam et al., 2007). Besides inflammation, the activated eosinophils are also implicated in airway remodeling, because they serve as a significant source of TGF-β_1 in asthmatic patients. TGF-β1 mediates airway remodeling by promoting the differentiation of fibroblasts to myofibroblasts that secrete extracellular matrix proteins (ECM), and stimulates airway smooth muscle hyperplasia via the Mitogen-activated protein kinase (MAPK) pathway (Kariyawasam et al., 2007; Halwani et al., 2011).

1.2.5 Lymphocytes

Asthma is more recognized as a T helper 2 (Th2) cell-mediated disease, because the production of Th2 cytokines (such as IL-4, IL-5 and IL-13) is responsible for the key pathophysiological features of asthma. Whereas IL-4 accounts for allergic sensitization and promotes Ig isotype switching in B cells to produce primarily IgE and IgG1, IL-5 is crucial for the presence of eosinophils, and IL-13 induces goblet cell hyperplasia (GCH) and airway hyperresponsiveness (Neveu et al., 2009). IL-25 is another Th2 cytokine described as a potentiator of Th2 memory responses. The finding that IL-25 activates eosinophils to produce a range of asthma-relevant mediators such as IL-8 suggests that IL-25 may play a pivotal role in maintaining Th2 central memory and sustaining asthmatic inflammation (Wong et al., 2005). Because of the widely-accepted Th1-Th2 paradigm, Th1 cells might be expected to be inhibitory of asthmatic airway inflammation. High levels of IFN-γ are expressed by Th1 cells, which have a critical

function in regulating the proliferation and apoptosis of T lymphocytes by upregulating the expression of Fas protein on T cell surfaces (Dalton et al., 2000; De Rose et al., 2004). It was demonstrated that apoptosis of Th1 cells, particularly in a high IFN-γ-producing fraction of T cells, is much more prominent in atopic patients compared with healthy controls as a mechanism of Th2 predominance (Akkoc et al., 2008). However, Th1 cells can become pathological super Th1 cells, which induce AHR and lung fibrosis by production of IFN-γ and IL-13 in the mouse model of bronchial asthma (Hayashi et al., 2007). Another T cell subset, regulatory T (Treg) cells, which act as the regulator of the effector class of the immune response, probably mediate the orchestration of Th1/Th2 balance whose defect could lead to the pathological expansion of Th2 cells in allergic asthma (Corthay A, 2009; Finotto, 2008). Other T cell subsets, such as Th17 cells, Th9 cells and Th22 cells have emerged and extended the existing Th1-Th2 paradigm. These T cells are the main members in the adaptive immune system and elicit unique responses to many immunological insults which they encounter. As depicted in Figure 1.2, the activated T cells produce specific cytokines, interact with other cells, and promote different types of inflammatory responses against different allergens. The variability and diversity of immune responses predict the complexity of asthma pathogenesis.

1.2.6 Neutrophils

Neutrophils play an immediate role in the immune defense against bacterial and fungal infections by a quick arrival at and a fast remove from inflammation sites (Monteseirín, 2009). Because of the immediate effect, neutrophils are not detectable at a later time point and no difference is found in the number of BAL neutrophils between asthmatic patients and normal control (Boulet et al., 1993). The role of neutrophils in the pathogenesis of asthma is underestimated. However, it should be noted that around 50% of asthmatic patients present noneosinophilic inflammation (Al Heialy et al., 2011; Monteseirín, 2009; Douwes et al., 2002). Most cases are associated with an increase of neutrophils in the airway submucosa. In chronic severe asthma, airway neutrophilia even becomes a recognised feature (Uddin et al., 2010). Because of the immediate effect,

neutrophils may be an ideal candidate for controlling the recruitment of other cell types (eg. dentritic cells) to the inflammation sites. Activated neutrophils release a set of chemokines, including IL-8 and growth-related oncogene α (GRO-α), which are chemoattractants for neutrophils. It forms a positive feedback loop for the accumulation of neutrophils (Monteseirín, 2009). Further studies are needed to explore the interactions between neutrophils and other cells and their integrated effect in the pathogenesis of asthma.

1.2.7 Dendritic cells

It is known that dendritic cells bridge innate and adaptive immunity in the pathogenesis of asthma (Lambrecht et al., 2009). In the lung, there are at least five subtypes of dendritic cells (DCs). They are distributed throughout the lung and trigger innate immune responses by taking up inhaled allergens. During the processing of allergens, DCs migrate to the draining lymph nodes. They express costimulatory molecules such as CD86 and high levels of MHC class II, which induce the proliferation of naïve T cells. They produce the chemokines CCL17 and CCL22, which bind to $CCR4^+$ Th2 cells and recruit them to inflammation sites. Lung DCs are the main antigen-presenting cells (APCs) which initiate Th2 immune response during airway inflammation. This has been demonstrated by using an OVA-induced asthma model, in which Th2 cells do not produce IL-4, IL-5 or IL-13 in the absence of $CD11c^+$ DCs (van Rijt et al., 2005).

1.2.8 Epithelial cells

The airway epithelium lining the upper airways acts as the interface between external and internal environments and provides the first line of defense against inhaled pathogens in innate immunity by producing inflammatory mediators and activating inflammatory cells. Epithelial cells are a major source for eotaxin, an eosinophil specific chemokine which recruits eosinophils from the blood into the lung. Eotaxin recognizes the sole receptor CCR3, which is expressed on eosinophils and Th2 cells involved in allergic responses. Together with IL-5, eotaxin makes up a two-step mechanism which

explains the development of tissue eosinophilia *in vivo* (Rothenberg et al., 1996). Epithelial cells can release KC (keratinocyte chemokine), also named CXCL1, which activates and recruits neutrophils within the airways contributing to the pathogenesis and severity of asthma and other respiratory diseases. Moreover, the notion that epithelial cells participate in asthma pathogenesis is reinforced by their secretion of CCL17 and TSLP (Thymic stromal lymphopoietin), which attract Th2 cells and promote Th2 cell differentiation, respectively (Heijink et al., 2007; Ying et al., 2005).

1.3 Innate and adaptive immunity in asthma

The initiation of allergen sensitivity occurs at mucosal contact surfaces, where innate immunity provides a powerful early defense. A broad range of innate immune cells participate in this defense, including epithelial cells, dendritic cells, macrophages, neutrophils and natural killer T cells. All these cells function to block the entry of microbes by recognizing innate molecule pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin receptors. The receptors recognize not only microbial products, but also cytokines like IFNγ, which enhances the ability to phagocytise microbes by innate immune cells. Adaptive immunity is also called acquired immunity, of which the two adaptive immune cells, T- and B-lymphocytes, are both implicated in asthma. T cells recognize antigens presented by antigen presenting cells (APC cells), and mediate cell immunity by releasing different cytokines. For example, Th17 cells secrete IL-17, a cytokine recognized as pro-inflammatory in allergic responses. The other T cells, Th1, Th2, Treg, Th9, and Th22 cells, which are briefly described in Chapter 1.2.5, contribute to asthma pathogenesis as well. Meanwhile, B cells mediate humoral immunity by producing antibodies such as IgE, which is suggestive of allergic immune response. It is becoming clear both innate and adaptive immunity have crucial roles in the development of asthma. In fact, innate and adaptive immunity are not mutually isolated, instead, they synergize with each other. On one hand, innate immunity directs the way that adaptive immune system responds to allergens. On the other hand, adaptive immunity combats microbes which have evaded innate immunity. The combined activation of both innate

and acquired immune response may account for a mixed inflammation of eosinophils and neutrophils in some patients of acute asthma (Douwes et al., 2002).

1.4 The "Hygiene hypothesis"

The "Hygiene hypothesis" was formulated in the case that allergic diseases have been increasing in the industrialized world with improved environmental living conditions. It was initially proposed by Strachan, who observed an inverse correlation between hay fever and the number of older siblings when following more than 17,000 British children born in 1958 (Strachan, 1989; Okada et al., 2010). The hypothesis states that modern health care and hygiene practices have led to a relative sterilization of the environment with diminished exposure to bacterial, viral or fungal components. Under such circumstances, the potential allergy-preventing ability of the microbial factors reduces or no longer exists. As a result, there is an imbalance of the immune system which predisposes individuals to the development of allergic diseases (Renz et al., 2006; Garn et al., 2007). This hypothesis was subsequently confirmed by several epidemiological studies and immunologic results. For example, in a study which assessed the relationship between exposure to pets in the early years of life and allergic sensitization, it was found that exposure to pets during infancy might have a protective effect later on in life (Hesselmar et al., 1999). In line with this point, antibiotic use in early childhood has been associated with an increased risk of developing asthma. The immunological explanation for this phenomenon is due to the dynamic nature of the immune system. In this system, immune response genes are more easily targeted and evolvable for the adaptation to a highly changeable and complexed environment than those involved in other biological processes such as cell growth (Sironi et al., 2010). The immune system is still under development in early life and appropriate exposure to allergic agents helps to shape the immune system. This evolutionary shaping role of allergens could also be shown in animal models. Nembrini and colleagues (Nembrini et al., 2011) have demonstrated that pulmonary exposure to the bacterium *Escherichia coli* (*E. coli*) leads to a suppression of allergic airway inflammation in OVA-sensitized mice. In addition to *E. coli*, other microbes such as *Bordetella pertussis* (Kim et al., 2004),

Chlamydia (Han et al., 2004), *Acinetobacter lwoffii* F78 (*A. lwoffii* F78) (Conrad et al., 2009; Brand et al., 2011) and *Lactococcus lactis* G121 (Debarry et al., 2007) have been shown to modulate allergic airway reactions or provide asthma protection.

1.5 Murine asthma models

Development of animal models is a feasible approach to explore the pathophysiology of diseases and potential discovery of novel therapeutics, since human experimentation is limited. Mice are the most common species studied in animal models of asthma. Mouse models of allergen exposure have been successfully designed to demonstrate the characteristic features of allergic asthma including airway inflammation, remodelling and airway hyperresponsiveness. Ovalbumin (OVA) derived from chicken egg is the most frequently used model allergen that induces a robust, allergic pulmonary inflammation in mice. Typically, it is injected intraperitoneally along with an adjuvant, aluminum hydroxide, which serves to improve the OVA's immunogenicity for reasons that are complex and not entirely understood (Bates et al., 2009; Kool et al., 2008). After the immune system has had a chance to mount a reaction against the antigen protein, which takes several days, the animal receives a further antigen exposure either directly to the lungs in the form of an aerosol or via postnasal drip following intranasal instillation. This elicits an inflammatory reaction in the lungs characterized by an influx of eosinophils, epithelial thickening and AHR, which has been demonstrated in a variety of studies. Although the OVA-induced allergic airway inflammation model can represent many of the central features of allergic asthma, there are still some shortcomings, as listed in Table 1.1. The relevance of ovalbumin to human asthma and the attempt to sensitize the mice through airways have led to the use of house dust mite (HDM) extract.

HDM represents one of the most common sources of aeroallergens worldwide. The mites are tiny (up to 0.3 mm) animals related to ticks and spiders, living in house dust and producing proteases which are highly allergic to the human being. Despite geographical differences, HDM allergens are related to 50-80% of asthmatics. The predominant species associated with human asthma are *Dermatophagoides*

pteronyssinus (*Der p*) and *Dermatophagoides farinae* (*Der f*), and their allergens have been divided into 19 groups (*Der p* 1-*Der p* 19, *Der f* 1-*Der f* 19). In recent years, HDM has been indicated of modeling asthma phenotype in different studies (Cates et al., 2004; Phipps et al., 2009) and has some advantages in mimicking human asthma (see Table 1.1). Compared to OVA, HDM is a more naturally occurring and clinically relevant allergen, since OVA is not related to inducing any type of human asthma. The sensitization of HDM is without additional adjuvant. It is possible to sensitize the mice through the respiratory tract, which is likely the route of sensitization in humans. It is also suggested that sensitizing the mice with HDM for a long time can emulate some features of chronic asthma while chronic OVA exposure may lead to inhalation tolerance (Stevenson et al., 2011; Swirski et al., 2002). Therefore HDM is a more suitable allergen for asthma models and also the concern of this study.

Table 1.1 Comparison of biological behaviors of OVA and HDM in murine asthma models

	OVA	**HDM**
Mode of sensitization	i.p., s.c.	i.n., IA
Dose usage	10 μg, 20 μg, 50 μg, 100 μg, 1000 μg	HDM extract: 25 μg, 50 μg, 100 μg Total protein: 2.5 μg, 5 μg, 10 μg, 20 μg, 40 μg, 80 μg
Adujvant	Alum, B. *pertussis*, etc	(-)
Inflammatory cell types	eosinophil, Th2 cell, Th1 cell, mast cell, dendritic cell, basophil	neutrophil, eosinophil, Th2 cell, Th17 cell, dendritic cell, basophil
Immunoglobulins	total IgE, OVA-specific IgE, IgG1, IgG2a	total IgE, HDM-specific IgE, IgG1
Lung histology	GCH	GCH
AHR	increased	increased

Note: i.p. refers to intraperitoneally; s.c. subcutaneuosly; i.n. intranasally; IA intratracheal aspiration; (-) no

1.6 Hypothesis and aim of this study

In recent years, exploration of the inverse relationship between microbial agents and allergic diseases becomes a hotspot in scientific research field. Several microbes including *Bordetella pertussis*, *Chlamydia*, *A. lwoffii F78* and *Lactococcus lactis* have been shown to provide asthma protection in OVA models, in which eosinophil airway recruitment is inhibited. Due to the low amount of neutrophils in OVA asthma models,

the microbial effect on neutrophil inflammation or a mixed inflammation of neutrophils and eosinophils is underappreciated. Since noneosinophilic asthma constitutes a considerable part of asthma, it is in urgent need to clarify the relationship between microbes and neutrophils. Therefore, the hypothesis of this study is that administration of the bacterium *Staphylococcus sciuri* (*S. sciuri*) inhibits murine asthma phenotype with an airway inflammation dominated by neutrophils and eosinophils through education of the immune system.

The aim in the first part of this study is to establish a murine asthma model with a mixed inflammation of airway neutrophils and eosinophils by using HDM extract. Although HDM has been universally regarded as a pivotal allergen, experimental data concerning asthma phenotype are still in controversy. One possible reason is that the biochemical properties of HDM extracts vary extensively among different preparations. The other explanation lies in the diverse doses of HDM extracts administrated in different studies. In this study, different origins, different doses of HDM extracts as well as different time points after the last sensitization of allergen are investigated in one mouse model successively. Through the examination of asthma-associated parameters, a detailed asthma phenotype will be delineated which consists of an inflammatory pattern, a profile of cytokine and chemokine expression and a subsequent change in serum immunoglobulins, lung histology and airway responsiveness. Furthermore, investigations of the underlying mechanisms for HDM-induced asthma phenotype are involved.

In the second part of this study, the Gram-positive bacterium *S. sciuri* will be employed as a possible preventive strategy in this mouse model. This is the first time to investigate the influential role of *S. sciuri* in HDM-induced asthma phenotype. The bacteria are isolated from the dust of a farm cowshed, which have been indicated of a protective role in childhood asthma (Ege et al., 2011). In these experiments, the bacteria are administrated prior to HDM sensitization. Corresponding biological events are recorded in kinetics and the influence of *S. sciuri* on HDM-phenotype is determined.

2 Materials and methods

2.1 Animals

For *in vivo* animal experiments, female wild type (WT) BALB/c mice aged 5-6 weeks were obtained from Harlan Winkelmann (Borchen, Germany). All mice were maintained under pathogen-free housing conditions. Water and food were supplied *ad libitum*. All experimental procedures were approved by the local animal ethics committee and met German and international guidelines. At least 4 mice were used per group.

2.2 Induction of asthma phenotype by HDM extract

House dust mite (HDM) extracts were obtained from Allergopharma Co. or Greer Labs. Mice were anaesthetized by using Ketamine/Rompun. According to protocol 1 (Figure 2.1), anesthetized mice received an intranasal instillation of PBS or HDM extract (50 µg or 100 µg of the whole extract dissolved in 50 µl PBS) on days 0, 7, 14, 21, 28, 35. Airway hyperresponsiveness was measured on day 36. 48 hours after the last challenge, the mice were sacrificed for further analysis.

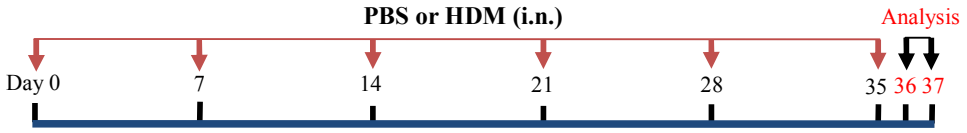


Figure 2.1 Protocol 1 for HDM-sensitized mouse model

According to protocol 2 (Figure 2.2), on days 0, 7, 14, 21, the mice received an intranasal instillation of PBS or 100 µg HDM extract (Allergopharma Co.) in 50 µl PBS. 24 and 48 hours after the final challenge, the mice were sacrificed respectively for further analysis.

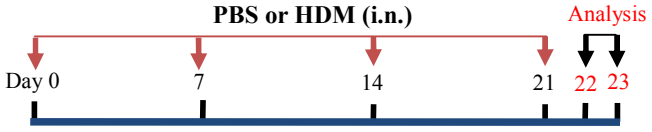


Figure 2.2 Protocol 2 for HDM-sensitized mouse model

Reagents:

House Dust Mite Extract (Lot no. W0657678)	Allergopharma Co, D
House Dust Mite Extract (Lot no. 125118)	Greer Labs, D
House Dust Mite Extract (Lot no. 140153)	Greer Labs, D
PBS (Dulbecco´s Phosphate Buffered Saline)	PAA Laboratoires GmbH, Cölbe, D

2.3 Treatment of *Staphylococcus sciuri* in HDM-induced asthma

For *Staphylococcus sciuri* (*S. sciuri*) treatment (protocol 3), the mice were given *S. sciuri* (10^8 CFU/25 µl) or PBS intranasally. It started nine days before the first sensitization, took place for 3 times in one week and lasted until the last challenge. 24 and 48 hours after the final challenge, the mice were sacrificed respectively for further analysis (see Figure 2.3).

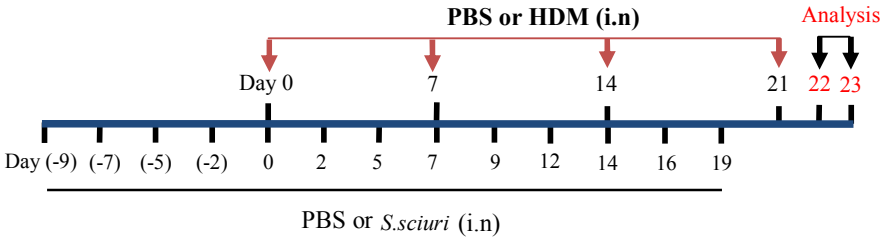


Figure 2.3 Protocol 3 for treatment of *Staphylococcus sciuri* in HDM-sensitized mouse model

Reagents:

Staphylococcus sciuri	Munich, D
House Dust Mite (Lot no. W0657678)	Allergopharmaco, D
PBS (Dulbecco´s Phosphate Buffered Saline)	PAA Laboratoires GmbH, Pasching, A

2.4 Measurement of airway hyperresponsiveness by Head-out body plethysmography

The Head-out body plethysmography measured standard lung function parameters like half-maximal expiratory and inspiratory flow, tidal volume, time of inspiration and expiration and respiratory rate on spontaneous breathing, non-anesthetized mice. Airway hyperresponsiveness (AHR) was measured one day after the last challenge of HDM extract and expressed as the concentration of methacholine (MCh_{50}) which

caused a 50% reduction in mid-expiratory airflow (EF_{50}).

The system consists of a glass Head-out body plethysmograph to which an aerosol exposure chamber is attached. For airflow measurement, the mice were positioned in the plethysmograph with the head sticking out of a neck collar (Φ 9 mm) into the ventilated exposure chamber. A calibrated pneumotachograph and a differential pressure transducer coupled to an amplifier were attached to the top port of the plethysmography. The respiratory movement from the mice will lead to a volume change in the plethysmograph and the resulting airflow is measured by the pneumotachograph. The amplified and digitized flow signals are intergrated with time to obtain tidal volume. From these data, the value of EF_{50} could be achieved by using the software Notocord hem 3.5.

The measurement started with the determination of the baseline level, during which the mice respired 4000 times without any substances delivered to the head exposure chamber. All the values obtained at this time were averaged and the mean value was taken as 100%. The corresponding differentiations to the control data were regarded as deviations from the baseline. The measurement was continued by the addition of bronchoconstrictor methacholine (MCh). MCh was dissolved in PBS for different concentrations: 0 mg/mL, 12.5 mg/mL, 25 mg/mL, 50 mg/mL, 75 mg/mL and 100 mg/mL. The solutions were delivered by a jet nebulizer to the head chamber every 5 minutes in an increasing dose, and every delivery lasted 70 seconds. The resulting airway constriction from administration of MCh induced characteristic changes in the tidal flow pattern, which were best revealed by a decrease in tidal midexpiratory flow (EF_{50}). Asthmatic mice are more sensitive to MCh compared to normal mice, thus with a lower MCh_{50} detected.

Reagents:

PBS (Dulbecco´s Phosphate Buffered Saline)	PAA Laboratoires GmbH, Cölbe, D
Methacholine	Sigma, Taufkirchen, D

Materials:

Glass exposure chamber	Forschungsstaetten, Medical School Hannover, D

Pneumotachograph PTM378/1.2	Hugo Sachs Elektronic, March-Hugstetten, D
Pressure Transducer 8T-2	Gaeltec, Dunvegan, Scotland
Amplifier HSE-IA	Hugo Sachs Elektronic, D
Notocord Hem 3.5 Software	Notocord, Paris, France

2.5 Acquisition of blood from animals

After the mouse was injected with 200 µl 5x anesthetics (50 mg/ml Ketamine 10 ml+ 2% Rompun 3.125 ml) intraperitoneally (i.p), skins under the axilla were cut out and around 0.5 ml blood from axillary vessels were collected. After coagulating at room temperature for one hour, the blood sample was centrifuged at 2000 rpm for 30 minutes at 4 °C. The serum was carefully transferred into a FINNTIP® 300 tube and stored at -20 °C until use.

Reagents:	
Ketamin Inresa	Inresa, Freiburg, D
2% Rompun	Bayer Health Care, Leverkusen, D
Materials:	
FINNTIP® 300	Thermo Scientific, Vantaa, F
Centrifuge Megafuge 1.0R	Heraeus, Osterode, D

2.6 Bronchoalveolar lavage (BAL)

The bronchoalveolar lavage (BAL) was used for the acquisition of cellular and protein components from the pulmonary airways.

2.6.1 Acquisition of BAL from animal experiments

After the mouse was sacrificed by injection of 5x anesthetics i.p, the trachea was dissected and cut into a small hole. Through the hole, a 1.3-mm-diameter venous catheter was inserted into the trachea and the lung was lavaged three times by 1 ml PBS with protease inhibitor. The cell suspension was centrifuged at 1200 rpm, 4 °C for 10 minutes and the cell-free supernatant was taken and stored at -20°C until further analysis. The cell pellet was resuspended with 1 ml 1% BSA in PBS and the number of total cells (leukocytes) was counted.

Reagents:

Ketamin Inresa	Inresa, Freiburg, D
2% Rompun	Bayer Health Care, Leverkusen, D
PBS (Dulbecco´s Phosphate Buffered Saline)	PAA Laboratoires GmbH, Pasching, A
Protease Inhibitor Cocktail	Roche, Mannheim, D
BSA, Fraction V	Roth, Karlsruhe, D

Materials:

Vasofix Venous Catheter	Braun, Melsungen, D
1 ml Syringe	BD, Madrid, S
Centrifuge Megafuge 1.0R	Heraeus, Osterode, D

2.6.2 Cell count and cell differentiation in the BAL

The total number of leukocytes was determined by using a CASY® TT cell counter. 50 µl volume was taken from each sample and made 1:200 dilution by CASY® Ton solution for cell counting. Before making cell differentiation, cells were adjusted to a concentration of 1×10^5/mL for cytospin preparations, and two cytospins were made for each sample by the following steps: 200 µl cell suspension was added into a sample chamber for one cytospin, centrifuged on Cytocentrifuge Cytospin 3 for 5 minutes at 700 rpm and the cells are centrifuged onto a microscope slide. The cytospins were air-dried at room temperature for 1 hour before they were stained with Diff-Quick solutions. The cell differentiation was carried out under light microscope at 400x magnification by using morphological criteria. In each slide, 100 leukocytes were counted for the differentiation of macrophages, eosinophils, neutrophils and lymphocytes. The absolute count of each cell, for example, eosinophil count was achieved by multiplying the total number of leukocytes with percentage of eosinophils.

Reagents:

CASY® Ton	Schärfe Systems, Reutlingen, D
Diff-Quick	Dade Behring, Eschborn, Germany

Materials:

CASY® Model TT (Cell-Counter)	Schärfe Systems, Reutlingen, D
Sample Chamber	Thermo Electron Corporation, USA
Cytocentrifuge Cytospin 3	GraphPad Software, La Jolla (CA), USA
Microscope Slides	Menzel GmbH, Braunschweig, D

Microscope (BH2)	Olympus, Hamburg, D

2.7 Lymphocyte restimulation

Lymphocyte restimulation was employed to assess immunological status of the body. To perform lymphocyte restimulation, lymph nodes were collected from animal experiments and pressed through cell strainers. The resulting cell suspension was restimulated with HDM extract *in vitro*.

2.7.1 Acquisition of lymph nodes from animal experiments

After the mice were sacrificed by injection of 5x anesthetics i.p, mediastinal lymph nodes were collected and put into ice-cold sterile medium (RPMI 1640+ 10% FCS+ 1% L-Glutamine + 1% Antibiotic/Antimycotic). The lymph nodes were pressed through a 100-µm-diameter cell strainer. The resulting cell suspension was poured into a 15 ml tube and filled with medium to a final volume of 5 ml. The tube was placed on ice until further processing.

Reagents:	
RPMI 1640	Schärfe Systems, Reutlingen, D
FCS Gold	PAA Laboratoires GmbH, Pasching, A
L-Glutamine	PAA Laboratoires GmbH, Pasching, A
Antibiotic/ Antimycotic	PAA Laboratoires GmbH, Pasching, A
Materials:	
Cell Strainer	BD Falcon, Bedford, USA
15ml Tube	Sarstedt, Nümbrecht, D
HERA safe	Heraeus, Osterode, D

2.7.2 *In vitro* restimulation of lymphocytes with HDM extract

The cell suspension from 2.7.1 was centrifuged at 1200 rpm for 10 minutes. The supernatant was removed and the cell pellet was gently agitated. 5 µl volume was taken from each sample and made 1:2000 dilution by CASY® Ton solution for cell counting. To get a cell concentration of 2×10^6/ml, different volume of medium was added to each sample according to the cell counts. 200 µl cell suspension from each sample was added into the well of 96 Well cell culture plate and stimulated with 50 µg/mL HDM extract.

To act as negative control, another 200 µl cell suspension was added into the well without HDM stimulation. After incubating at a condition of 37 °C and 5% CO_2 for 72 hours, the plate was centrifuged at 1200 rpm, 4 °C for 10 minutes. Cell-free supernatant was carefully transferred and stored at -20 °C until subsequent analysis.

Reagents:

RPMI 1640	Schärfe Systems, Reutlingen, D
FCS Gold	PAA Laboratoires GmbH, Pasching, A
L-Glutamine	PAA Laboratoires GmbH, Pasching, A
Antibiotic/ Antimycotic	PAA Laboratoires GmbH, Pasching, A

Materials:

Centrifuge Megafuge 1.0R	Heraeus, Osterode, D
96 Well Cell Culture Plate	Cellstar, D
HERA cell 240	Heraeus, Osterode, D
HERA safe	Heraeus, Osterode, D

2.8 Lung histology

Lung histology provided morphological changes in the lung. To observe lung histology, lung perfusion, acquisition and histological staining and analysis were performed.

2.8.1 Lung perfusion

To avoid possible interference of red blood cells in the following examinations, such as RNA isolation and histological staining, lung perfusion was performed during animal preparation. The mouse was sacrificed by administration of 5x anesthetics. Chest cavity was opened and the trachea, the lung and the heart were detached. After acquisition of the BAL, the venous catheter was left in the trachea. The arteries were then severed, a 1.3 mm catheter needle was inserted into the right atrium and at least 3 ml PBS was injected slowly through the heart and into the lung, taking red blood cells out of the severed arteries until the lung turned white.

Reagents:

PBS Dulbecco	Biochrom AG, Berlin, D

Materials:

Catheter needle	Braun, Melsungen, D

2.8.2 Acquisition of lung tissue from animal experiments

After perfusion, the right lung was tied off at the main-stem bronchus, cut out and put into liquid nitrogen for RNA isolation. The left lung was tied off at the trachea after injection of 1 ml cold formalin through the trachea catheter, then cut out and fixed in 6% paraformaldehyde via the trachea for 24 hours.

Reagents:

Paraformaldehyde	Merck, Darmstadt, D

Materials:

Micro-crew Tube 2 ml	Sarstedt, Nümbrecht, D

2.8.3 Histological staining for the lung

The fixed left lung was taken out of the paraformaldehyde, embedded in 2% agarose and cut into 2 mm segments. All the segments were put on one layer, filled with 2% agar and embedded with paraffin. 5 μm sections were cut from the paraffin blocks by using Rotary Microtome and loaded onto microscope slides.

Each slide was set to dry in a 60 °C incubator overnight and then deparaffinized through xylene, rehydrated in descending alcohol series (100%, 96% and 70%), and rinsed in distilled water before histological staining was performed.

Periodic acid-Schiff (PAS) was used for demonstration of mucus production and goblet cell hyperplasia in the airways. The deparaffinzed slide was immersed in periodic acid, which selectively oxidized glucose residues to aldehydes. After rinsing, the slide was immersed in Schiff's reagent, which was bound by aldehydes and a magenta-color reaction was created. Because mucus is a glycoprotein produced by goblet cells, they are stained purple-magenta in PAS staining. The slide was dipped into Hematoxylin solution acc. to Gill III for a counterstain followed by a thorough rinsing in water. The slide was dehydrated in ascending alcohol series (70%, 96% and 100%), fixed in xylene and covered with Vectormount.

Reagents:

Agar	Merck, Darmstadt, D
Paraffin	Merck, Darmstadt, D
Ethanol	Sigma-Aldrich Chemie GmbH, Steinheim,D
Periodic acid	Merck, Darmstadt, D
Hematoxylin solution acc. to Gill III	Roth, Karlsruhe, D
Schiff's reagent	Roth, Karlsruhe, D

Materials:

Rotary Microtome	Microm GmbH, Walldorf, D
Microscope Slides	Menzel GmbH, Braunschweig, D
Mounting Medium	Vector Laboratories, Inc. Burlingame, CA
Incubator	Heraeus, Osterode, D

2.8.4 Analysis of histological changes in the lung

Histological observations were carried out under a PC-based Olympus light microscope BX51 equipped with a CAST-Grid system. All sections were delineated (at a magnification of 25x) and the fields of view to be analysed (at a magnification of 400x) were automatically defined according to systemic uniform random sampling. Around 150 fragments were selected per lung section.

In PAS-stained lung sections, goblet cells and epithelial cells which were crossed by basement membrane and the mucus which hit the test points were counted. The percentage of goblet cells (%) and the volume of GC-mucus content (µm) were calculated according to the following formula (from lab SOP document):

$$P_{GC}\,(\%) = \frac{N_{GC}\cdot 100}{N_{GC}+N_{EC}} \qquad V_{Mucus}\,(\mu m) = \frac{N_{Mucus}\cdot L(P)\cdot 0.5}{N_{GC}+N_{EC}}$$

where P_{GC} is percentage of goblet cells, N_{GC} the number of goblet cells, N_{EC} the number of epithelial cells, V_{Mucus} the volume of GC-mucus content, N_{Mucus} the number of GC-mucus content, and *L(P)* is the length per test point.

Materials:

Microscope Slides	Menzel GmbH, Braunschweig, D
Microscope (BX51)	Olympus, Hamburg, D
CAST	Visiopharm, DK

2.9 Enzyme-linked immunosorbent assay (ELISA)

ELISA served to quantitate the presence of antigens or antibodies in the samples. The basic principle of ELISA is to use an enzyme to detect the binding of unknown antigens (or antibodies) to known antibodies (or antigens). To detect antigen A, purified first antibody binds to the surface of microtiter plate. Unspecific bindings are blocked by adding irrelevant proteins. The samples were coated onto the surface of the wells. Antigen A from the samples bound to the first antibody. Unbound substances were washed away. After the adding of the biotinylated second antibody immunocomplex was formed. This was recognized by HRP (Horseradish Peroxidase) and therefore became Streptavidin-Peroxidase-Complex. The addition of substrate will cause a color reaction and the optical density was immediately determined by using a microtiter-plates-reader in which the absorption wavelength is set to 450 nm.

2.9.1 ELISA for cytokines and chemokines in the BAL fluid (BALF)

The ELISA procedure for cytokine IL-5 was detailed as the following:

The primary antibody for IL-5 was diluted with 0.1 M $NaHCO_3$ solution (pH=8.3) to get a concentration of 1 µg/mL. A 96-well-microtiterplate was coated with 50 µl/well diluted primary antibody, and incubated at 4°C overnight. The solution was discarded and the plate was washed with washing buffer (PBS with 0.1% Tween 20) for four times. Free binding sites were saturated by incubating the plate with 100 µl/well blocking buffer (PBS with 1% BSA) at room temperature for 2 hours. After washing, a series of 2-fold diluted standards (the highest standard is 4000 pg/mL), blanks and samples were added to the plate and incubated on the shaker at room temperature for 2 hours. The plate was washed four times and 50 µl detection antibody was applied to each well with a concentration of 2 µg/mL. The plate was placed on a shaker and incubated for 2 hours at room temperature. After another four-time washing step, the streptavidin-peroxidase was 1:1000 diluted and added into the plate, followed by 30 minutes' incubation at room temperature in the dark. After a final eight-time washing, the plate was applied with 100 µl/well peroxidase substrate solution and incubated in the dark for about 15

minutes' color reaction. The addition of 50 µl stop solution (2 M H_2SO_4) into each well caused the color changed from blue to yellow, which was measured by a microtiter-plates-reader with the absorption wavelength set to 450 nm. The data analysis was carried out by using Software Megallan 5. A standard curve was generated to verify analysis linearity and the concentration of IL-5 was calculated and expressed as pg/mL.

The detections of IFN-γ, IL-10, IL-17, CXCL1, CXCL2, and CCL11 (eotaxin) in the BAL followed the same procedure as described above, but with different concentrations of antibodies and standards (see Table 2.1).

Table 2.1 Concentrations of antibodies and standards for the cytokine- and chemokine-ELISA

Item	Primary Antibody	Detection Antibody	1st Standard
IL-5	1 µg/mL	2 µg/mL	4000 pg/mL
IFN-γ	3 µg/mL	1 µg/mL	10,000 pg/mL
IL-10	1:250 diluted	1 µg/mL	4000 pg/mL
IL-17	2 µg/mL	400 ng/mL	1000 pg/mL
CXCL1	2 µg/mL	200 ng/mL	1000 pg/mL
CXCL2	2 µg/mL	75 ng/mL	1000 pg/mL
Eotaxin	0.8 µg/mL	200 ng/mL	500 pg/mL

Reagents

Purified rat anti-mouse/human IL5	BD Pharmingen, San Diego, USA
Biotinylated rat anti-mouse IL-5	BD Pharmingen, San Diego, USA
Purified rat anti-mouse IFN-γ	BD Pharmingen, San Diego, USA
Biotinylated anti-mouse IFN-γ	BD Pharmingen, San Diego, USA
BD OptEIA™ Set Mouse IL-10	BD Pharmingen, San Diego, USA
Mouse IL-17	R&D Systems, Minneapolis, USA
Mouse CXCL1/KC	R&D Systems, Minneapolis, USA
Mouse CXCL2/MIP2	R&D Systems, Minneapolis, USA
Mouse CCL11 (eotaxin)	R&D Systems, Minneapolis, USA
PBS (Dulbecco´s Phosphate-buffered Saline)	Biochrom AG, Berlin, D
BSA, Fraction V	Roth, Karlsruhe, D
Tween 20	Roth, Karlsruhe, D
Streptavidin/Peroxidase	Sigma, Taufkirchen, D
BM Blue POD Substrate	Roche, Mannheim, D
$NaHCO_3$	Merck, Darmstadt, D

H_2SO_4	Merck, Darmstadt, D
Materials:	
96-Well-Microtiterplate	Nunc, Wiesbaden, D
Nunc-Immuno^{TM} Wash 12	Nunc, Wiesbaden, D
Microtiter-plates-reader Sunrise	Tecan, Crailsheim, D
Magellan 5	Tecan, Crailsheim, D

2.9.2 ELISA for immunoglobulins in the blood serum

The level of HDM-specific IgE (HDM-IgE) in the blood serum was determined by the following steps:

HDM extract was diluted to 50 µg/mL in 0.1 M $NaHCO_3$ solution (pH=8.3). A 96-well-microtiterplate was coated with 50 µl/well diluted HDM solution, and incubated at 4 °C overnight. The solution was discarded and the plate was washed with washing buffer for four times. Free binding sites were saturated by incubating the plate with 100 µl/well blocking buffer at room temperature for 2 hours. After washing, blood samples were 1:10 diluted in washing buffer and added to the plate and incubated on the shaker at room temperature for 2 hours. The plate was washed four times and 50 µl detection antibody was applied to each well with a concentration of 2.5 µg/mL. The plate was placed on a shaker and incubated for 2 hours at room temperature. After another four-time washing, the streptavidin-peroxidase was 1:1000 diluted and added into the plate, followed by 30 minutes' incubation at room temperature in the dark. After a final eight-time washing, the plate was applied with 100 µl/well peroxidase substrate solution and incubated in the dark for about 15 minutes' color reaction. The addition of 50 µl stop solution (2 M H_2SO_4) into each well led to a color change from blue to yellow, which was measured by a microtiter-plates-reader with the absorption wavelength set to 450 nm. The data analysis was carried out by using Software Megallan 5. The value of HDM-specific IgE was expressed as OD_{450} (Abs-Blank), in which Abs stands for absorption.

The same procedures were applied to the detections of HDM-specific IgG1 (HDM-IgG1), HDM-specific IgG2a (HDM-IgG2a), but the concentrations of antibodies

for each item differed (see Table 2.2). The measurement of total IgE followed a similar procedure but with known concentrations of primary antibody, detection antibody and standards from which a standard curve was formed and the concentration of total IgE was calculated and expressed as ng/mL.

Table 2.2 Concentrations of antibodies for immunoglobulin-ELISA

Item	Primary Antibody	Detection Antibody	1st Standard	Sample Dilution
HDM-specific IgG1	/	2.5 µg/mL	/	1:100
HDM-specific IgG2a	/	2.5 µg/mL	/	1:100
HDM-specific IgE	/	2.5 µg/mL	/	1:10
Total IgE	10.6 µg/mL	2.5 µg/mL	250 ng/mL	1:10

Reagents

HDM (Lot no. W0657678)	Allergo Pharmaco, D
Anti-mouse IgG1 biotinylated	BD Pharmingen, San Diego, USA
Anti-mouse IgE biotinylated	BD Pharmingen, San Diego, USA
Anti-mouse IgG2a biotinylated	BD Pharmingen, San Diego, USA
PBS (Dulbecco´s Phosphate-buffered Saline)	Biochrom AG, Berlin, D
BSA, Fraction V	Roth, Karlsruhe, D
Tween 20	Roth, Karlsruhe, D
Streptavidin/Peroxidase	Sigma, Taufkirchen, D
BM Blue POD Substrate	Roche, Mannheim, D
$NaHCO_3$	Merck, Darmstadt, D
H_2SO_4	Merck, Darmstadt, D

Materials:

96-Well-Microtiterplate	Nunc, Wiesbaden, D
Nunc-Immuno™ Wash 12	Nunc, Wiesbaden, D
Microtiter plates reader Sunrise	Tecan, Crailsheim, D
Analysis software Magellan	Tecan, Crailsheim, D

2.10 Cytometric Bead Array (CBA)

The CBA is a bead based immunoassay for the simultaneous determination of different cytokines in one single sample. It combines the principles of sandwich immunoassays with the fluorescence detection ability of flow cytometry. Briefly, the

capture beads which are coated with specific capture antibodies are mixed with a sample or standard and then mixed with phycoerythrin (PE) conjugated detection antibodies to form sandwich complexes. After incubation and subsequent washing, the samples are acquired by a flow cytometry and the data are transformed to graphical and tabular formats by FCAP ArrayTM Software.

A cytokine kit of FlowCytomix Mouse Th1/Th2 8 plex from Bender MedSystem was used for the detection of IL-4, IL-5, IL-13, IFN-γ, IL-10, IL-17A, IL-9, GM-CSF, in the supernatants of *in vitro* restimulated lymphocytes. A volume of 25 µl is required for each sample.

Reagents:	
FlowCytomix Mouse Th1/Th2 8 plex	Bender MedSystems, Wien, A
Materials:	
BD FACSArray™	BD, Heidelberg, D
FCAP Array™ Software	BD, Heidelberg, D

2.11 Analysis of gene expression at mRNA level

Quantitative real time polymerase chain reaction (QPCR) was employed to quantify gene expression in mRNA level. To carry out QPCR, RNA isolation and quantification, DNA digestion and reverse transcription were performed.

2.11.1 RNA isolation

The isolation of RNA from lung homogenates was performed by using a combination of reagents from TriFast and RNeasy Mini kit. First, 50~100 mg tissue was taken from the lung and put into liquid nitrogen. After nitrogen vaporized, the tissue was disrupted and homogenized into powder by a pestle followed by the adding of 1 ml TriFast reagent. The reagent contains phenol and guanidine in a single phase solution. The addition of chloroform and subsequent centrifugation separate the homogenate into three phases, with RNA in the colorless upper phase. 350 µl RNA solution was carefully transferred into RNeasy Mini column together with the same volume of 70% ethanol. In the column, RNA was selectively bound to a silica-gel membrane and contaminants

were efficiently washed away by RNeasy buffers. High-quality RNA was then eluted in 30 µl RNase-free water and stored in 1.5 ml Eppendorf tube at -80 °C until further use.

Reagents:

peqGOLD TriFast	Peqlab, Erlangen, D
RNeasy Mini kit	Qiagen, Hilden, D
Chloroform	Roth, Karlsruhe, D
Ethanol absolute	Sigma-Aldrich Chemie GmbH, Steinheim,D
RNase free water	Eppendorf, Hamburg, D

Materials:

BIOFUGE pico	Heraeus, Osterode, D
1.5ml Eppendorf Tube	Eppendorf, Hamburg, D

2.11.2 RNA quantification

The determination of RNA concentration was carried out by using a Spectrophotometer. 2 µl volume was taken from each sample and made 1:50 dilution by RNase-free water. RNA content was determined by measuring absorpance at 260 nm. The ratio of 260/280 was obtained by simultaneous measurement at 280 nm, followed by the statement about the quality of RNA preparation. In good preparations, this ratio ranges from 1.8 to 2.0.

Reagents:

RNase free water	Eppendorf, Hamburg, D

Materials:

Ultrospec 3100 pro UV/Visible Spectrophotometer	Biochrom, Cambridge, UK

2.11.3 DNA digestion

DNA digestion was performed to remove the contaminating DNA from the RNA preparations, by using the enzyme deoxyribonuclease I (DNase I). After acquiring a solution of 0.5 µg RNA in 8 µl H_2O, 1.2 µl 10x DNase Buffer, 1 µl DNase I and 1.2 µl H_2O were added into the solution to make a volume of 12 µl, and incubated at room temperature for 15 minutes. The reaction of DNA degradation was stopped by the addition of 1 µl 25 mM EDTA. DNase I was inactivated by ten minutes' incubation at 65 °C. The DNA-free RNA was then stored at -80 °C until further use.

Reagents:

Deoxyribonuclease I, Amplification Grade	Invitrogen, Carlsbad, CA, USA

Materials:

Thermocell Cooling & Heating Block	Biozym, Hessisch Oldendorf, D

2.11.4 Reverse transcription

In the process of reverse transcription, the purified mRNA was transcribed into complementary DNA (cDNA) by using reverse transcriptase. The reaction system includes 0.5 µg RNA, 2 µl 10x RT buffer, 2 µl dNTP mix (final concentration: 0.5 mM of each dNTP), 1 µl oligo-dT 18 primer (final concentration: 1 µM) and 1 µl Omniscript reverse transcriptase, with an end volume of 20 µl filled with RNase-free water. The reaction condition is one hour's incubation at 37 °C followed by denaturing at 93 °C for 5 minutes. The resulting cDNA was diluted 1:2 with RNase-free water and stored at -20 °C until subsequent analysis.

Reagents:

Omniscript® Reverse Transcription Kit	Qiagen, Hilden, D
Oligo-dT 18 Primer	Metabion, Martinsried, D

Materials:

MJ Mini™ Personal Thermal Cycler	Bio-Rad, Singapore

2.11.5 Quantitative real time Polymerase Chain Reaction (QPCR)

The principle of QPCR is based on real-time detection of fluorescent signals from amplified DNA. The machine (RotorGene™ 3000) used for this detection consists of PCR device and fluorescence detector. PCR amplification was performed by using QuantiTect SYBR Green PCR kit, which contains a TaqPolymerase from MasterMix, dNTPs, PCR buffer and the fluorescent dye SybrGreen I. SybrGreen I intercalated into the double-stranded DNA (dsDNA) and gave a fluorescent signal which is received by the detector. During the extension phase, more and more SybrGreen I bound to the newly synthesized dsDNA, and by this way the increase of PCR products were monitored real-time. After 45 cycles, melting curve analysis was performed to verify PCR products. Because every dsDNA had a unique melting temperature (Tm), a

temperature at which half of the DNA helical structure is lost, and a narrow melting peak, one can judge from the melting curve if any unspecific products were produced during amplification.

The reaction system includes 5 µl SybrGreen mix, 0.25 µl forward primer (10 pmol/µl), 0.25 µl reverse primer (10 pmol/µl) and 2.5 µl RNase-free water. The reaction condition was listed in Table 2.3, and the annealing temperature was adjusted for optimal amplification in each reaction.

To determine the difference at mRNA expression level, targeted genes were amplified simultaneously with the house keeping gene L32 or β-actin. The targeted genes included TNFα, TLR4, GM-CSF, CCL17, IL-4, IL-5, IL-13, IFNγ, IL-10, IL-17A, CXCL1, CXCL2, CCL11 (eotaxin), GATA-3, Tbx21, Foxp3, Rorγt. Calculation of relative changes in the targeted genes was performed against L32 or β-actin by using the ΔCT method (Zasłona et al., 2009). Primer sequences are detailed in Table 2.4.

Table 2.3 Reaction conditions for QPCR

PCR amplification			
Pre-denaturing	15 minutes	95 °C	
Denaturing	15 seconds	94 °C	45 Cycles
Annealing	30 seconds	55 °C	
Extension	15 seconds	72 °C	
Melting curve analysis			
Temperature gradient from 65 °C to 95 °C with an interval of 0.1 °C			

Table 2.4 (to be continued) Primers for QPCR

Gene Name		Sequence (5'-3')
TNF-α	sense	AgC CCA CgT CgT AgC AAA CC
	antisense	TAC AAC CCA TCg gCT ggC AC
TLR4	sense	gCA ggT ggA ATT gTA TCg CC
	antisense	TTC gAg gCT TTT CCA TCC AA
GM-CSF	sense	ATg CCT gTC ACg TTg AAT gA
	antisense	CCg TAg ACC CTg CTC gAA TA
CCL17	sense	AAT gTA ggC CgA gAg TgC Tg
	antisense	CAT CCC Tgg AAC ACT CCA CT

Table 2.4 (continued) Primers for QPCR

Gene Name		Sequence (5'-3')
IL-4	sense	TCA ACC CCC AgC Tag TTg TC
	antisense	TgT TCT TCg TTg CTg TgA gg
IL-5	sense	CTg TCC TCg CCA CAC TTC
	antisense	ACg ATg Agg CTT CCT gTC
IL-13	sense	CCA TCC CAT CCC TAC AgA AA
	antisense	ATA ggC AgC AAA CCA TgT CC
IFNγ	sense	gCg TCA TTG AAT CAC ACC Tg
	antisense	TgA gCT CAT TgA ATg CTT gg
IL-10	sense	gCA Tgg CCC AgA AAT CAA gg
	antisense	TCT TCA CCT gCT CCA CTg CC
IL-17A	sense	AgC Tgg ACC ACC ACA TgA AT
	antisense	AgC ATC TTC TCg ACC CTg AA
CXCL1	sense	CTT gAA ggT gTT gCC CTC Ag
	antisense	Tgg ggA CAC CTT TTA gCA TC
CXCL2	sense	AAA gTT TgC CTT gAC CCT gA
	antisense	Agg CAC ATC AggG TAC gAT CC
CCL11 (eotaxin)	sense	CCA Agg ACT Tgg CTT CAT gT
	antisense	AAC TCg TCC CAT TgT gTT CC
GATA-3	sense	gTC ATC CCT gAg CCA CAT CT
	antisense	Agg gCT CTg CCT CTC TAA CC
Tbx21	sense	Agg TgT CTg ggA AgC TgA gA
	antisense	CCA CAT CCA CAA ACA TCC Tg
Foxp3	sense	TCC AAT CCC TgC CCT TgA CC
	antisense	CAC ATC ATC gCC Cgg TTT CC
Rorγt	sense	ATA gCA CTg ACg gCC AAC TT
	antisense	AgA AAC Tgg gAA TgC AgT
L32	sense	gCA AgT TCC Tgg TCC ACA AT
	antisense	ggg ATT ggT gAC TCT gAT gg
β-actin	sense	TgT TAC CAA CTg ggA CgA CA
	antisense	ggg gTg TTg AAg gTC TCA AA

Reagents:	
QuantiTect™ Sybr Green PCR Master Mix	Qiagen, Hilden, D
Primers	Metabion, Martinsried, D
Materials:	
RotorGene™ Software	Corbett, Sydney, AUS
0.1 mL Strip-Tubes	Corbett, Sydney, AUS
RotorGene™ 3000	Corbett, Sydney, AUS

2.12 Statistical analysis

These data are representative of experiments with 4-6 mice in each group. All data are expressed as mean ± standard error (SEM) and analyzed for significance using Student's unpaired t-test or one-way ANOVA. Student's unpaired t-test was used to compare the difference between two groups and calculate the probability value. To compare more than two groups, one-way ANOVA was conducted. A probability value of less than 0.05 ($p < 0.05$) was considered to indicate statistical significance. Data analysis was performed by using the software GraphPad Prism 5.

3 Results

3.1 Comparison of different origins of house dust mite extracts in a mouse model

In the first experiment, three different commercial HDM extracts (provided by Allergopharma Co. and Greer Labs, respectively), namely HDM1, HDM2, and HDM3 were compared in regards to inducing an asthma phenotype. HDM extracts are complicated mixtures of proteins and peptides, among which *Der p* 1 and *Der p* 2 are regarded as the major allergens. One difference between the three used extracts is the content of *Der p* 1. For example, in HDM1 which was provided by Allergopharma Co., *Der p* 1 (4.5 µg) constitutes 4.5% of the whole extract weight (100 µg), while in Greer Labs-origined HDM2, *Der p* 1 constitutes 1.4% of 100 µg extract. The concentrations of *Der p* 1, *Der p* 2 and total protein in the three extracts are summarized in Table 3.1. According to sensitization protocol 1 (Figure 2.1), the whole HDM extracts were applied intranasally to the mice once a week for six times, each time at a dose of 100 µg.

Table 3.1 A brief data sheet of HDM extracts

Name	Company	Batch No.	*Der p 1* content	*Der p* 2 content	Protein content
HDM1	Allergopharma	W9001009	4.49 µg/100 µg	2.35 µg/100 µg	29 µg/100 µg
HDM2	Greer Labs	125118	1.4 µg/100 µg	unknown	27 µg/100 µg
HDM3	Greer Labs	140153	1.3 µg/100 µg	unknown	23 µg/100 µg

3.1.1 AHR and airway inflammation induced by different origins of HDM extracts

As shown in Figure 3.1, administration of the three HDM extracts didn't affect airway reactivity of the lung (A), but had different influences on the recruitments of inflammatory cells into the airways (B). In comparison to control mice (PBS-treated mice), HDM1-immunized mice displayed a prominent airway inflammation which was due to an increase in the number of neutrophils, eosinophils and lymphocytes. In HDM2-immunized mice, the number of total leukocytes and neutrophils were significantly higher, eosinophils and lymphocytes remained comparable, and

macrophages were significantly lower, as compared to PBS-mice. In contrast, sensitization of the mice with HDM3 only promoted a weak inflammatory response.

A strong airway inflammation was observed in both HDM1- and HDM2-immunized mice, but the inflammatory types varied. In HDM1-mice, neutrophils and eosinophils were the predominant infiltrating cells, while in HDM2-mice, a more robust neutrophil influx was induced into the airway, which was not accompanied by eosinophil inflammation.

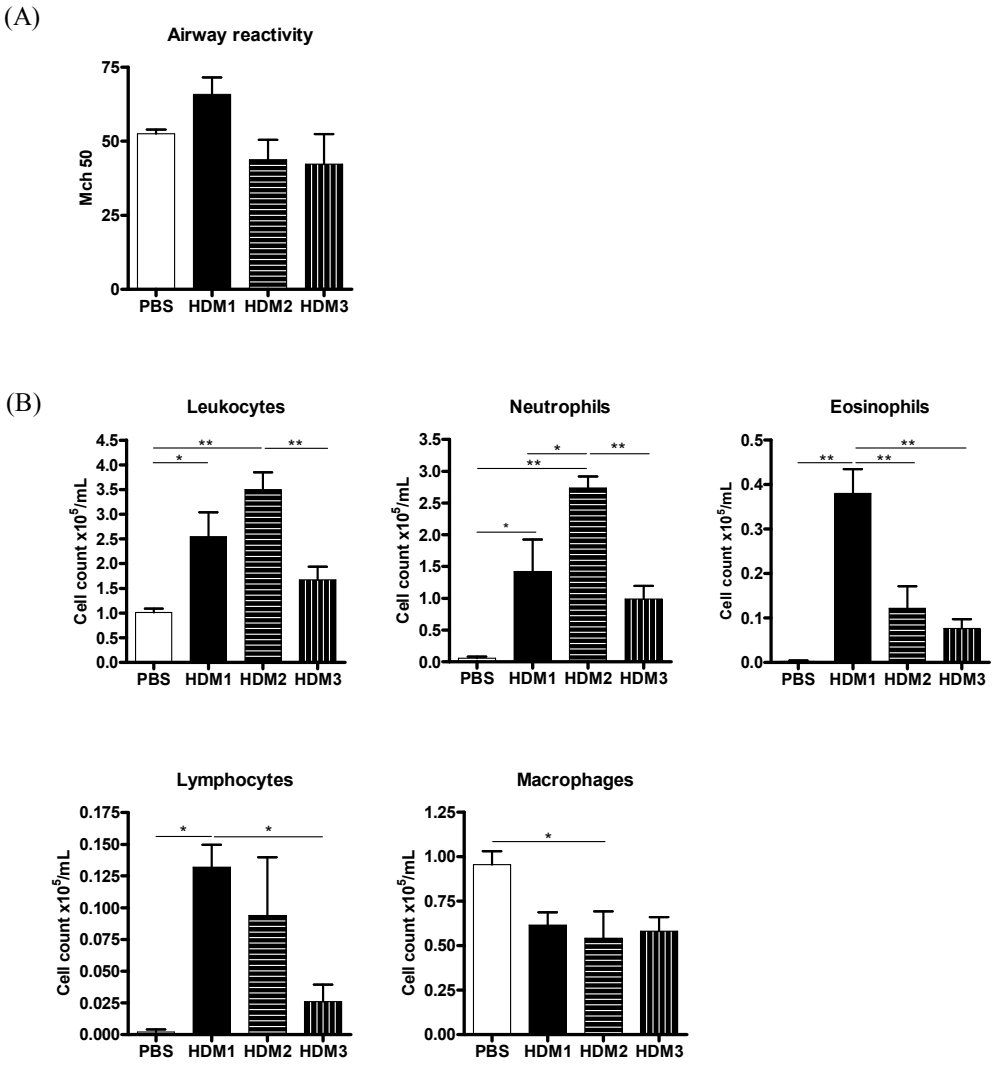


Figure 3.1 Comparison of BAL cell types in a murine model sensitized by different origins of HDM extracts

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM extracts once a week for 6 times. (A) 24 hours after the last instillation, aiway reactivity was measured by Head-out body plethysmography. (B) 48 hours after the last instillation, cell differentiation of leukocytes in the BAL fluid was performed. $^{*}p < 0.05$, $^{**}p < 0.01$

3.1.2 Cytokines and chemokines in the BAL fluid

Cellular airway inflammation was closely related to dysfunctions in Th2 proinflammatory cytokines (Busse et al., 2001) or chemokines. Therefore, various cytokines and chemokines in the BAL fluid were determined. As demonstrated in Figure

3.2, the protein levels of CXCL1 were significantly elevated in mice when sensitized by the three HDM extracts, as compared to PBS-mice. Meanwhile, the chemokine CXCL2 and the cytokines IL-5, IFNγ and IL-17 were undetectable in all mice.

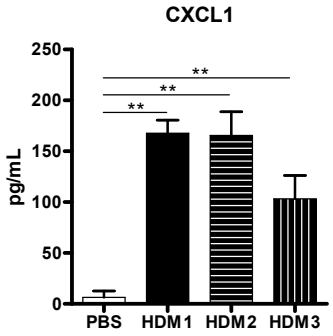


Figure 3.2 Comparison of BAL CXCL1 in a murine model sensitized by different origins of HDM extracts

BALB/c mice (n = 5 per group) were instilled intranasally with 100 μg HDM extracts once a week for 6 times. 48 hours after the last instillation, the protein level of chemokine CXCL1 in the BAL fluid was examined by ELISA. $^{**}p < 0.01$

3.1.3 Immunoglobulins in the blood serum

The production of total IgE in the blood serum was not affected by the sensitizations of the three HDM extracts (see Figure 3.3).

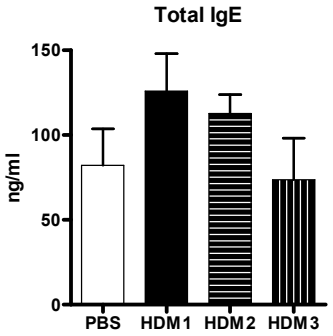


Figure 3.3 Comparison of total IgE in a murine model sensitized by different origins of HDM extracts

BALB/c mice (n = 5 per group) were instilled intranasally with 100μg HDM extracts once a week for 6 times. 48 hours after the last instillation, the level of total IgE in the blood serum was examined by ELISA.

3.1.4 Histological observations in the lung tissue

Histological examination remains the "gold standard" for assessing asthmatic airway remodeling such as goblet cell hyperplasia (GCH) and mucus hypersecretion. As depicted in Figure 3.4, in mice sensitized by the three HDM extracts, the percentage of goblet cells were markedly increased. The volume of mucus content was concurrently increased without reaching statistical difference.

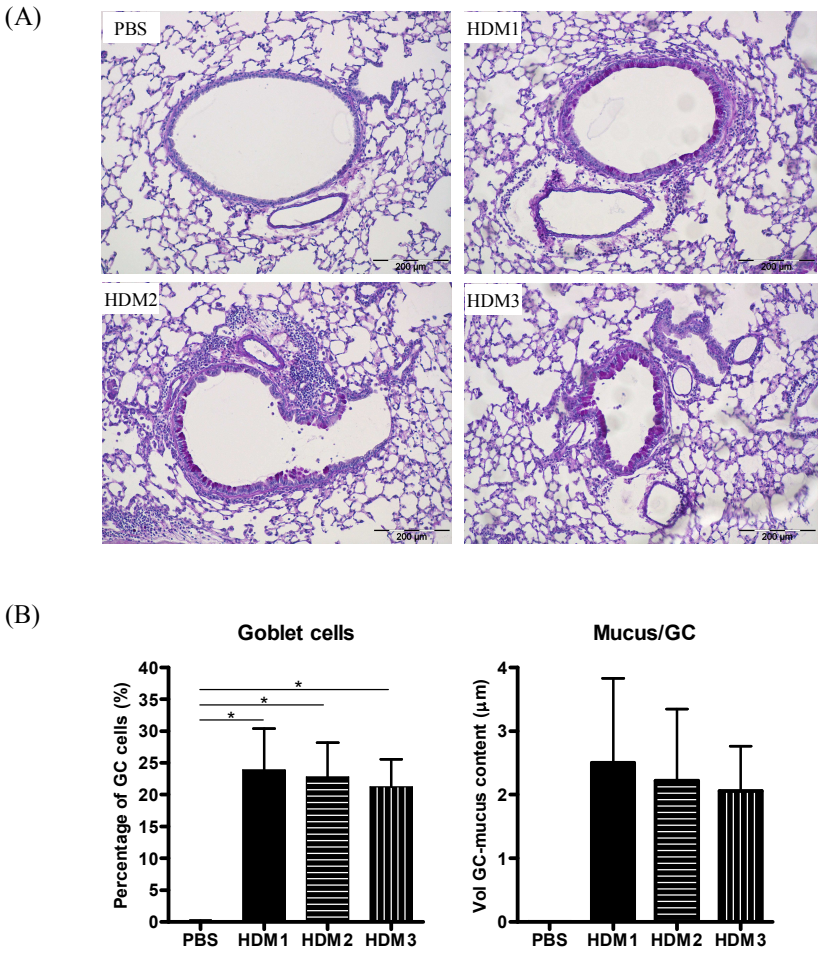


Figure 3.4 Comparison of histological observations in a murine model sensitized by different origins of HDM extracts

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM extracts once a week for 6 times. 48 hours after the last instillation, the lungs were cut into 5 µm sections and subjected to PAS staining. (A) Representative photomicrographs of PAS-stained lung sections. (B) Proportion of goblet cells and the volume of mucus content were determined by using the software CAST. $^{*}p < 0.05$

3.2 Comparison of different doses of HDM extracts in the induction of asthma phenotype in BALB/c mice

It is known that the dose of the sensitizing allergen is a critical determinant for inducing an asthma phenotype in the murine OVA models. Could a lower dose of HDM extract still elicit an airway inflammation dominated by neutrophils and eosinophils as described above? To address this question, the mice were sensitized with different doses of HDM1 (10 μg, 50 μg and 100 μg), according to sensitization protocol 1. After six times of sensitization once a week, the mice were sacrificed and asthma-associated parameters were analyzed. Initial experiment demonstrated that 10 μg HDM1 did not induce an asthmatic response in the mouse model (data not shown). In this experiment, dose effect of HDM1 was determined by comparing various parameters between 50 μg and 100 μg HDM1-treated mice. The extract HDM1 was provided by Allergopharma Co. (see Table 3.2).

Table 3.2 A brief data sheet of HDM1

Name	Company	Batch No.	*Der p* 1 content	*Der p* 2 content	Protein content
HDM1	Allergopharma	W0657678	4.49 μg/100 μg	2.35 μg/100 μg	29 μg/100 μg

3.2.1 AHR and airway inflammation elicited by different doses of HDM extrats

Compared with the PBS group, neither application of the mice with 50 μg or 100 μg HDM1 significantly changed airway reactivity of the lung (see Figure 3.5 (A)). However, as shown in Figure 3.5 (B), an inflammatory response of the airway was elicited by the instillation of HDM1, which was mainly due to a rise in the number of neutrophils and eosinophils. The lymphocytes increased slightly, without reaching a statistical difference. This response was more pronounced in mice sensitized by 100 μg HDM1 than in mice sensitized by 50 μg HDM1.

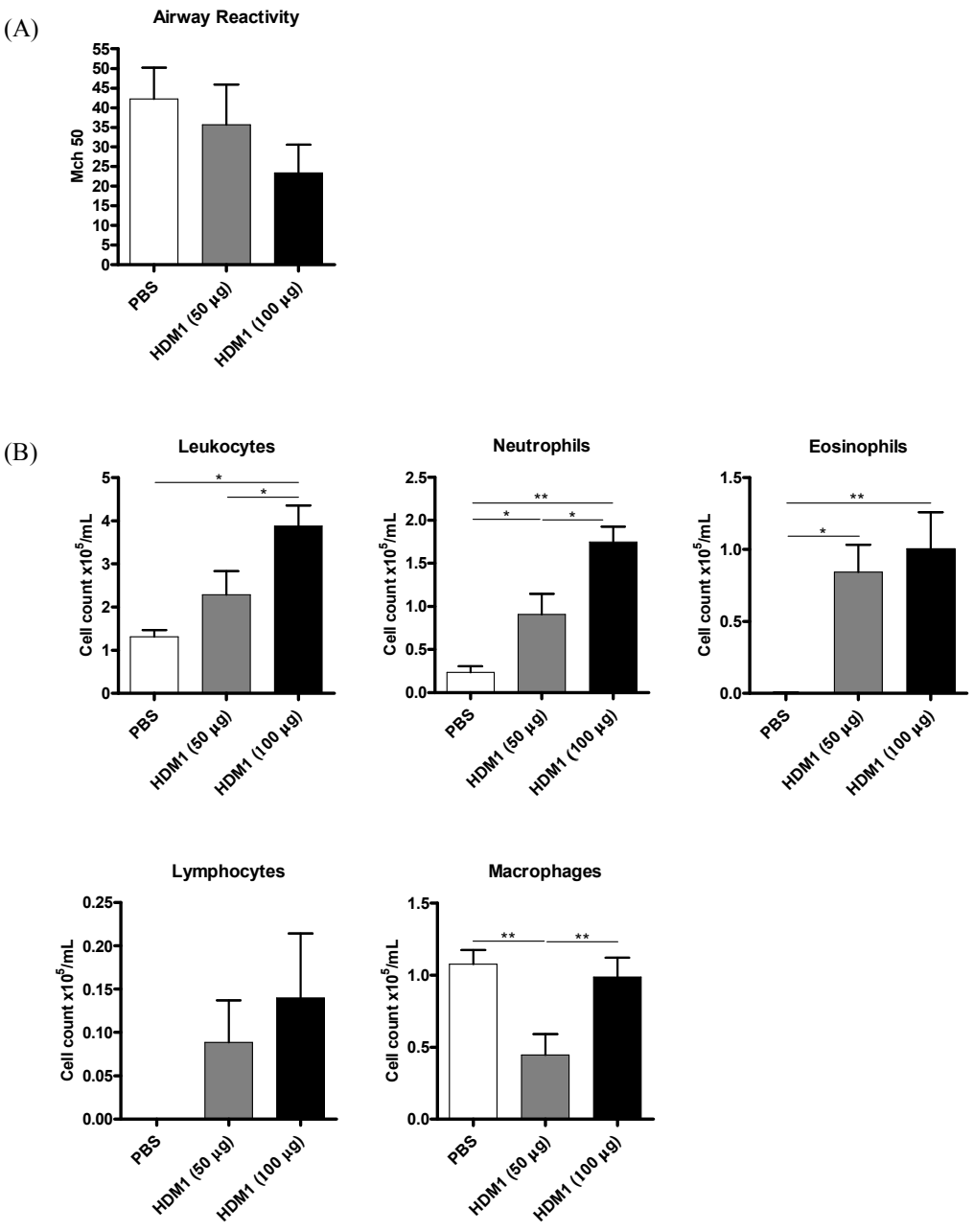


Figure 3.5 Comparison of BAL cell types in a murine model sensitized by different doses of HDM1

BALB/c mice (n = 6 per group) were instilled intranasally with 50 µg or 100 µg HDM1 once a week for 6 times. (A) 24 hours after the last instillation of HDM1, aiway reactivity was measured by Head-out body plethysmography. (B) 48 hours after the last instillation of HDM1, cell differentiation of leukocytes in the BAL fluid was performed. $^{*}p < 0.05$, $^{**}p < 0.01$

3.2.2 Cytokines and chemokines in the BAL fluid

Different doses of HDM1 resulted in different concentrations of cytokines and chemokines in the BAL fluid. As shown in Figure 3.6, in mice sensitized by 100 μg HDM1 the protein levels of CXCL1 and IL-5 were substantially elevated. In mice sensitized by 50 μg HDM1, the level of CXCL1 was substantially elevated as well, whereas the level of IL-5 was subtly increased, without reaching statistical difference. The levels of the chemokine CXCL2 and the cytokines IFNγ and IL-17 were undetectable in all mice.

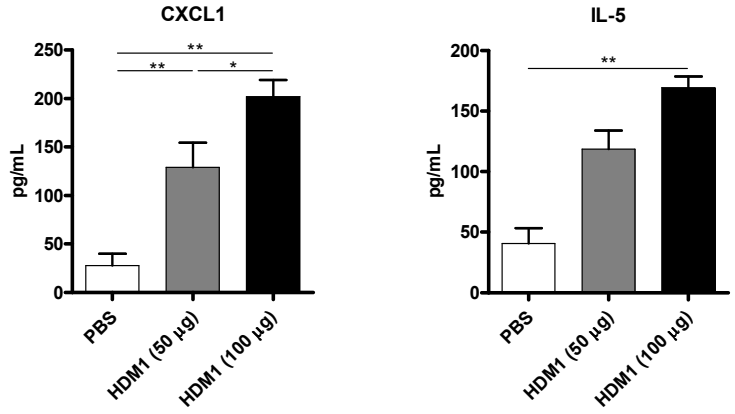


Figure 3.6 Comparison of BAL CXCL1 and IL-5 in a murine model sensitized by different doses of HDM1

BALB/c mice (n = 6 per group) were instilled intranasally with 50 μg or 100 μg HDM1 once a week for 6 times. 48 hours after the last instillation, protein levels of CXCL1 and IL-5 in the BAL fluid were examined by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$

3.2.3 Immunoglobulins in the serum

As shown in Figure 3.7, in mice sensitized by 100 μg HDM1, the level of HDM-IgG1 was markedly increased, the level of HDM-IgG2a was moderately increased ($p = 0.067$), and the level of total IgE remained comparable to that of PBS-mice. In mice sensitized by 50 μg HDM1, the level of HDM-IgG1 was significantly increased as well, the level of HDM-IgG2a was not altered, while the level of total IgE was elevated, almost statistically differently ($p = 0.063$). In contrast, the

amount of HDM-IgE was undetectable in all groups.

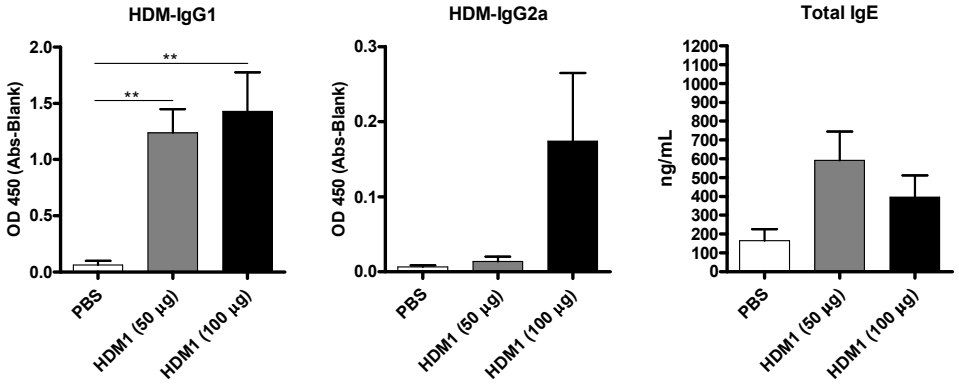


Figure 3.7 Comparison of immunoglobulins in a murine model sensitized by different doses of HDM1

BALB/c mice (n = 6 per group) were instilled intranasally with 50 µg or 100 µg HDM1 once a week for 6 times. 48 hours after the last instillation, the levels of serum immunoglobulins HDM-IgG1, HDM-IgG2a, and total IgE were examined by ELISA. $^{**}p < 0.01$

3.2.4 Histological manifestations in the lung tissue

As depicted in Figure 3.8, administration of 50 µg or 100 µg HDM1 elicited a substantial increase in the proportion of goblet cells and the volume of mucus content. Between the two HDM groups, there was no apparent difference regarding GCH and mucus hypersecretion.

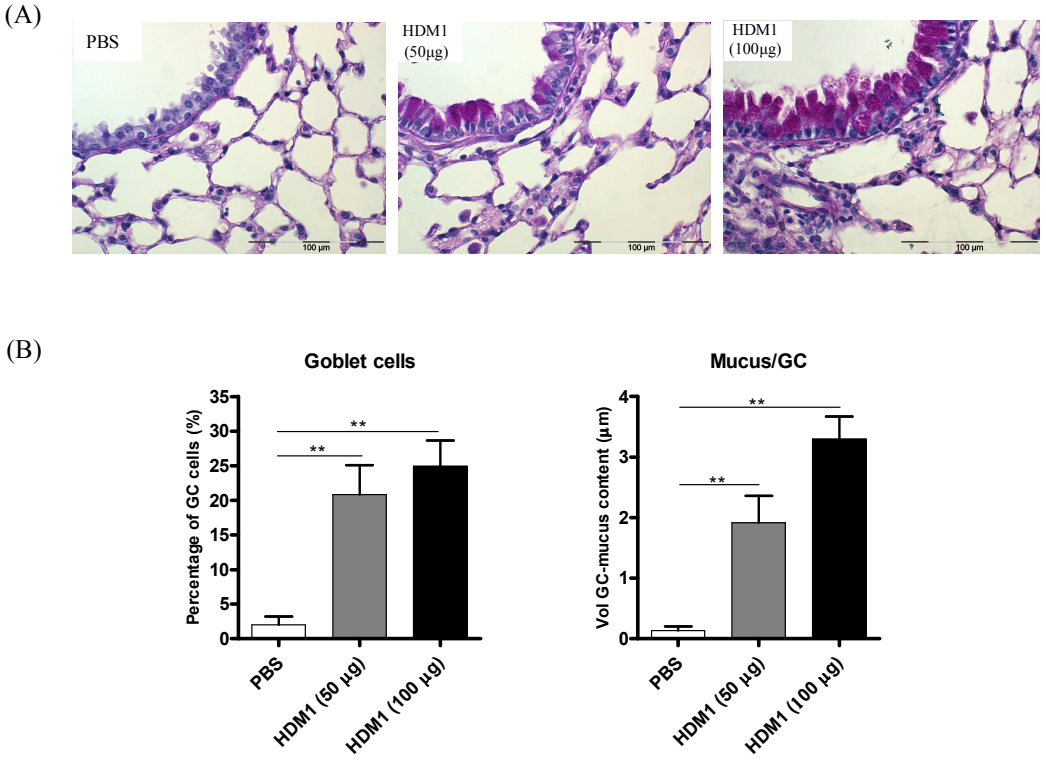


Figure 3.8 Comparison of histological manifestations in a murine model sensitized by different doses of HDM1

BALB/c mice (n = 6 per group) were instilled intranasally with 50 μg or 100 μg HDM1 once a week for 6 times. 48 hours after the last instillation, the lungs were cut into 5 μm sections and subjected to PAS staining. (A) Representative photomicrographs of PAS-stained lung sections. (B) Proportion of goblet cells and the volume of mucus content were determined by using the software CAST. $^{**}p < 0.01$

3.2.5 Expression profiling of asthma-related genes in the lung tissue

To further investigate the underlying mechanism which accounted for the abnormal biological events in HDM-induced asthma phenotype, expression profiling of asthma-related genes was carried out. First, the expressions of innate immune genes such as TNFα, TLR4, GM-CSF and CCL17 were examined. Then, genes which encode the cytokines and chemokines IL-4, IL-5, IL-13, IFNγ, IL-10, IL-17, CXCL1, CXCL2 and CCL11 (eotaxin) were analyzed. Afterwards, transcription factors including GATA-3, Tbx21, FOXp3 and Rorγt which regulate T cell balance were quantified.

As shown in Figure 3.9 and Figure 3.10, a variety of genes showed aberrant mRNA expression in response to HDM1. In mice sensitized by 100 μg HDM1, the expressions of TNF-α, CCL17, IL-5, IL-13, IL-10, IL-17A, CXCL1, and CCL11 (eotaxin) were markedly upregulated; the expressions of IL-4 (p = 0.105) and TLR4 (p = 0.074) were moderately enhanced; the expressions of GM-CSF, IFNγ, CXCL2, GATA3, Tbx21, Foxp3 and Rorγt remained unaltered, as compared to PBS-mice. In mice sensitized by 50 μg HDM1, the expressions of TNF-α, IL-13, IL-10, IL-17A and CCL11 (eotaxin) increased significantly, the expressions of IL-4 (p = 0.105) and TLR4 (p = 0.074) were moderately enhanced, and the expressions of GM-CSF, CCL17, IL-5, IFNγ, CXCL1, CXCL2, GATA3, Tbx21, Foxp3 and Rorγt remained comparable to PBS-mice.

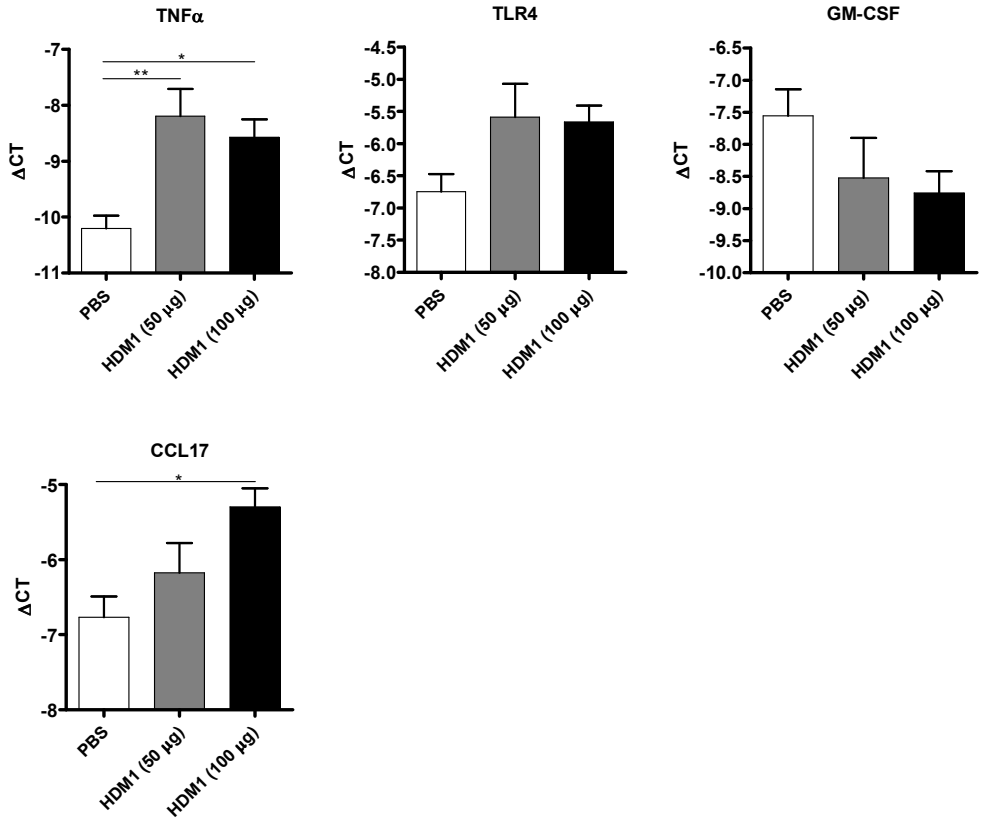


Figure 3.9 Comparison of mRNA expressions of innate immune genes in a murine model sensitized by different doses of HDM1

BALB/c mice (n = 6 per group) were instilled intranasally with 50 μg or 100 μg HDM1 once a week for 6 times. 48 hours after the last instillation, the lungs were homogenized and mRNA expressions of genes TNFα, TLR4, GM-CSF and CCL17 were analyzed by QPCR. $^{*}p < 0.05$, $^{**}p < 0.01$

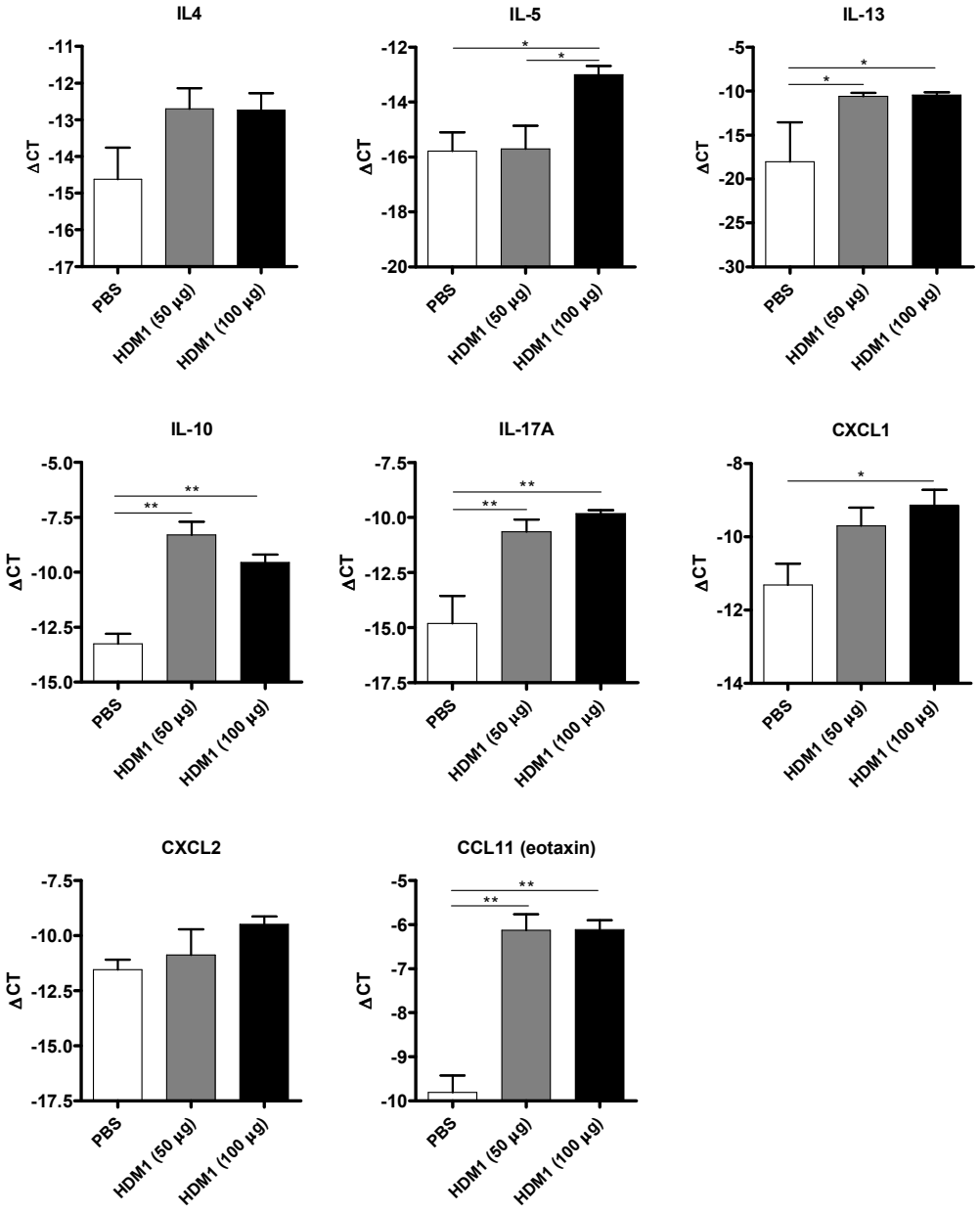


Figure 3.10 Comparison of mRNA expressions of cytokines and chemokine genes in a murine model sensitized by different doses of HDM1

BALB/c mice (n = 6 per group) were instilled intranasally with 50 µg or 100 µg HDM1 once a week for 6 times. 48 hours after the last instillation, the lungs were homogenized and mRNA expressions of genes IL-4, IL-5, IL-13, IL-10, IL-17, CXCL1, CXCL2 and CCL11 (eotaxin) were analyzed by QPCR. $^{*}p < 0.05$, $^{**}p < 0.01$

3.3 Evaluation of the kinetics of HDM-induced asthmatic response in BALB/c mice

Based on reported data and our previous results, this experiment was designed to investigate crucial biological events 24 hours and 48 hours after the last instillation of HDM1. The sensitization was carried out according to sensitization protocol 2 (Figure 2.2). The dose used in this experiment was 100 µg HDM1, which was provided by Allergopharma Co. (see Table 3.3).

Table 3.3 A brief data sheet of HDM1

Name	Company	Batch No.	*Der p* 1 content	*Der p* 2 content	Protein content
HDM1	Allergopharma	W0657678	4.49 µg/100 µg	2.35 µg/100 µg	29 µg/100 µg

3.3.1 Kinetics of development of airway inflammation

It was noticed that inflammatory cells migrated into the airway successively at different time points. As depicted in Figure 3.11, 24 hours after the last instillation of HDM1, the sensitized mice showed a sharp increase in the number of total leukocytes, mostly due to an increase of the neutrophils, while the numbers of lymphcotyes and macrophages remained unchanged, as compared to PBS-mice. At this time point, no eosinophils appeared in HDM-sensitized mice or PBS-mice. Instead, a rise in eosinophils was observed 48 hours after the last instillation. Also at this time point, the number of lymphocytes increased significantly, the numbers of total leukocytes and neutrophils were still higher, while macrophages remained unaltered, as compared to PBS-mice.

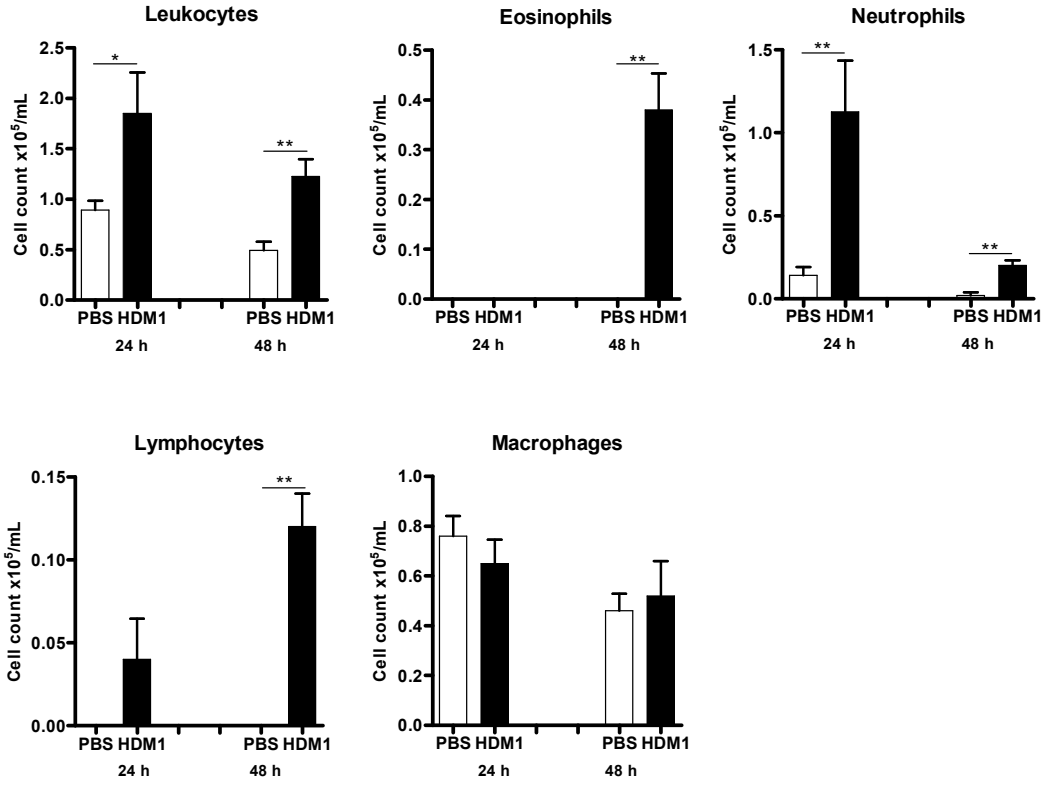


Figure 3.11 Kinetics of development of airway inflammation in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. 24 hours and 48 hours after the last instillation of HDM1, the mice were sacrificed, respectively, and cell differentiation of leukocytes in the BAL fluid was performed. $^{*}p < 0.05$, $^{**}p < 0.01$

3.3.2 Kinetics of production of BAL cytokines and chemokines

As shown in Figure 3.12, 24 hours after the last instillation of HDM1, the sensitized mice displayed a remarkable increase of protein levels of CXCL1 and IL-5 in the BAL fluid, as compared to PBS-mice. 48 hours after the last instillation, the level of chemokine CXCL1 remained higher in HDM-sensitized mice than in PBS-mice, while the protein level of IL-5 in the sensitized mice returned to a normal level of nearly zero. In contrast, the chemokine CCL11 (eotaxin) was not detectable in the BAL fluid at both time points in HDM-sensitized and non-sensitized mice.

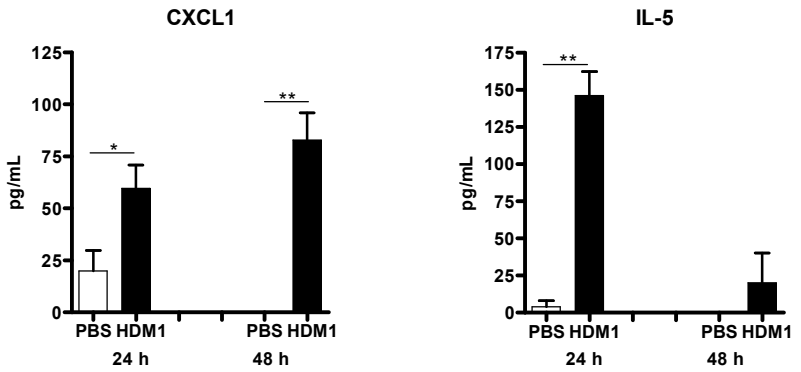


Figure 3.12 Kinetics of BAL CXCL1 and IL-5 concentrations in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. 24 hours and 48 hours after the last instillation of HDM1, the mice were sacrificed, respectively, and protein levels of CXCL1 and IL-5 in the BAL fluid were determined by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$

3.3.3 Kinetics of production of immunoglobulins in the serum

No distinguishable differences were found in the titers of serum HDM-IgG1, HDM-IgG2a and total IgE between HDM-sensitized mice and control mice at both time points.

3.3.4 Kinetics of histological observations in the lung tissue

The sensitized mice developed goblet cell hyperplasia and mucus hypersecrection 24 hours after the last instillation of HDM1, which continued at the second time point, 48 hours after the last instillation (Figure 3.13).

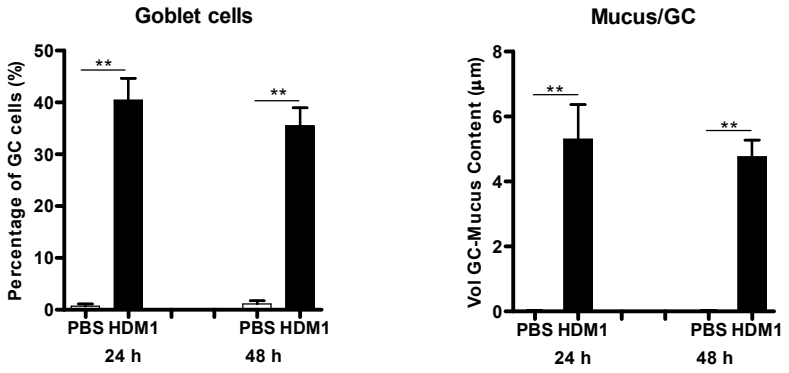


Figure 3.13 Kinetics of histological observations in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. 24 hours and 48 hours after the last instillation of HDM1, the mice were sacrificed, respectively, and proportion of goblet cells and the volume of mucus content were determined by use of microscopic examination and analysis of the software CAST. $^{**}p < 0.01$

3.3.5 Differential cytokine production from *in vitro* restimulated lymphocytes

Kinetic assessment of cytokine production of HDM-restimulated lymphocytes revealed that between the two time points, lymphocytes from HDM-sensitized mice had a stronger immune activity at the first time point. As shown in Figure 3.14, lymphocytes from the sensitized mice produced a greater amount of IL-4, IFNγ, IL-10, IL-17A and GM-CSF 24 hours after the last instillation of HDM1. Meanwhile, the production of IL-5, IL-13 and IL-9 showed a rising tendency at the first time point, as compared to the second time point. The above mentioned cytokines were not inducible when the lymphocytes from PBS-mice were stimulated with HDM *in vitro*.

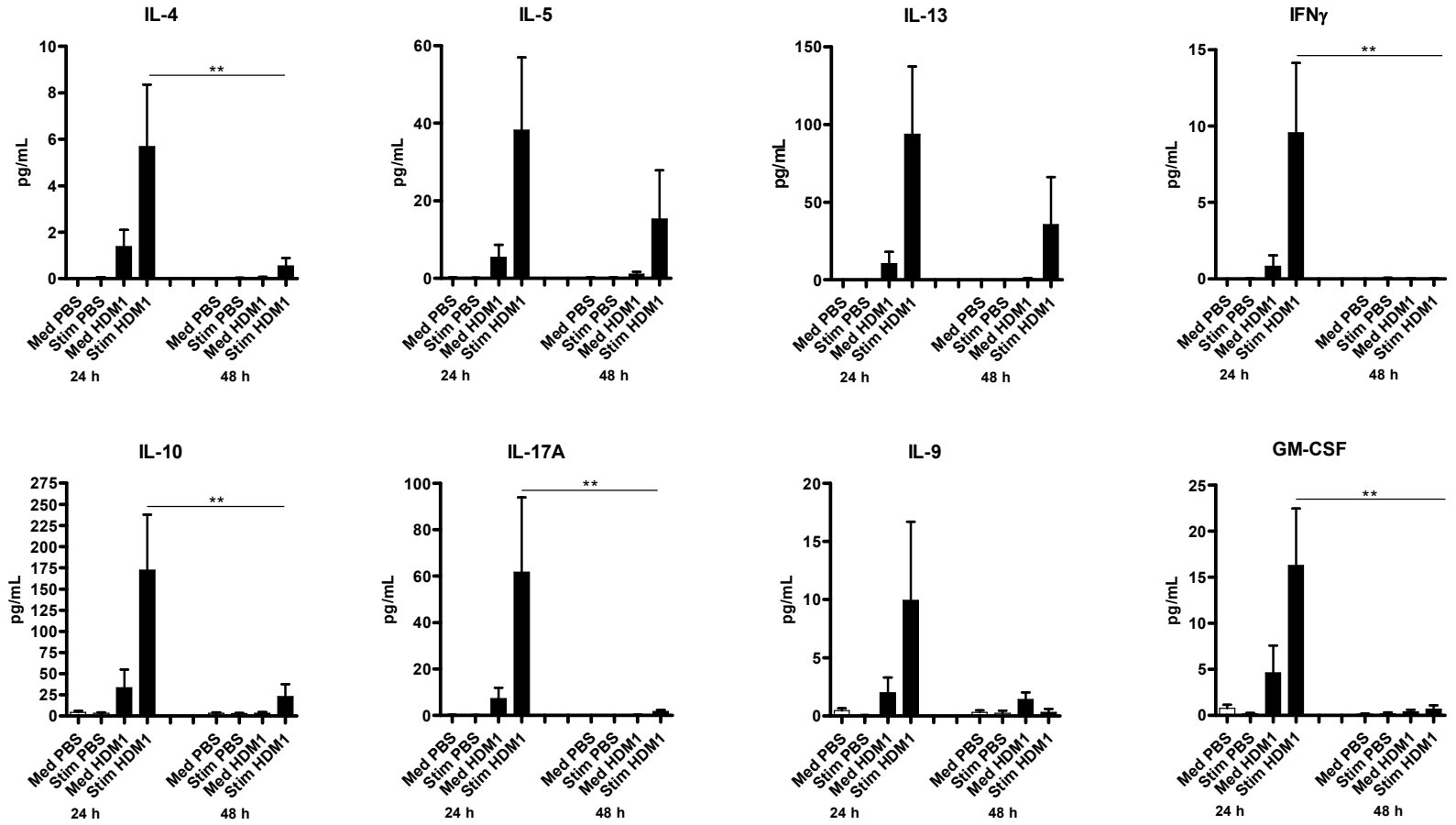


Figure 3.14 Differential cytokine productions from *in vitro* restimulated lymphocytes in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 μg HDM 1 once a week for 4 times. 24 hours and 48 hours after the last instillation of HDM1, the mice were sacrificed, respectively. Mediastinal lymphocytes were harvested and restimulated with 50 μg/ml HDM1 for 72 hours *in vitro*. The levels of cytokines IL-4, IL-5, IL-13, IFNγ, IL-10, IL-17A, IL-9 and GM-CSF in the cultured supernatant were determined by using CBA multiplex. To serve as a negative control, lymphocytes were incubated with medium. $^{*}p < 0.05$, $^{**}p < 0.01$

To summarize, in the first part of this study, investigating the influence of different origins, doses and time points of HDM extracts on the outcome of the asthmatic phenotypes demonstrated the following findings: HDM1 from Allergopharma Co. elicited a distinct asthma phenotype with a mixed airway inflammation, an increased production of serum HDM-IgG1 and a subsequent histological change. This phenotype was more pronounced in mice sensitized by 100 µg HDM1 than 50 µg HDM1. The most prominent feature of this phenotype was that neutrophils were present in parallel with eosinophils and constituted the major component of airway inflammation. Neutrophils and eosinophils are activated at different time points by different chemotactic factors and their activations might represent different biological events.

3.4 *S. sciuri* abolished asthma phenotype in HDM-induced asthma model

In this experiment, the Gram-positive bacterium *S. sciuri* was applied as a preventive strategy for HDM-induced asthma phenotype. According to sensitization protocol 3 (Figure 2.3), the mice received *S. scirui* and HDM1 via the airways. 24 hours and 48 hours after the last instillation of HDM1, asthma-related parameters were compared between HDM-mice with *S. sciuri* treatment and without *S. sciuri* treatment. The bacterial strain *S. sciuri* was isolated from the dust of a farm cowshed and 10^8 CFU were used for each treatment. HDM1 was provided by Allergopharma Co. (see Table 3.4) and 100 µg of the whole extract was given to the mice for each sensitization.

This was the first time to investigate the influential role of *S. sciuri* in HDM-induced asthma model. It should be mentioned that various biological events at the two check points in HDM-mice were demonstrated in Chapter 3.3. In this chapter, focus lies on phenotypic changes in HDM-sensitized mice when treated with *S. sciuri*. To exclude potential pathogenicity from *S. sciuri*, mice only treated with the bacterium served as negative control.

Table 3.4 A brief data sheet of HDM1

Name	Company	Batch No.	*Der p* 1 content	*Der p* 2 content	Protein content
HDM1	Allergopharma	W0657678	4.49 µg/100 µg	2.35 µg/100 µg	29 µg/100 µg

3.4.1 *S. sciuri* attenuated airway inflammation in HDM-induced murine asthma model

As shown in Figure 3.15 (A), sensitization of the mice with HDM1 led to a significant rise in airway reactivity, which was not reversed by administration of *S. sciuri.* However, HDM-induced airway inflammation was attenuated by the bacterium *S. sciuri*. As shown in Figure 3.15 (B), at the first time point, 24 hours after the last instillation of HDM1, administration of *S. sciuri* significantly reduced the neutrophil cell count in HDM-sensitized mice. At this time point, administration of the bacterium did not change the counts of eosinophils, lymphocytes, or macrophages in HDM-sensitized mice. At the second time point, 48 hours after the last instillation, the

count of neutrophils as well as that of eosinophils in HDM-sensitized mice was markedly reduced by the bacteria, while the counts of lymphocytes and macrophages in HDM-mice were not changed by the bacteria.

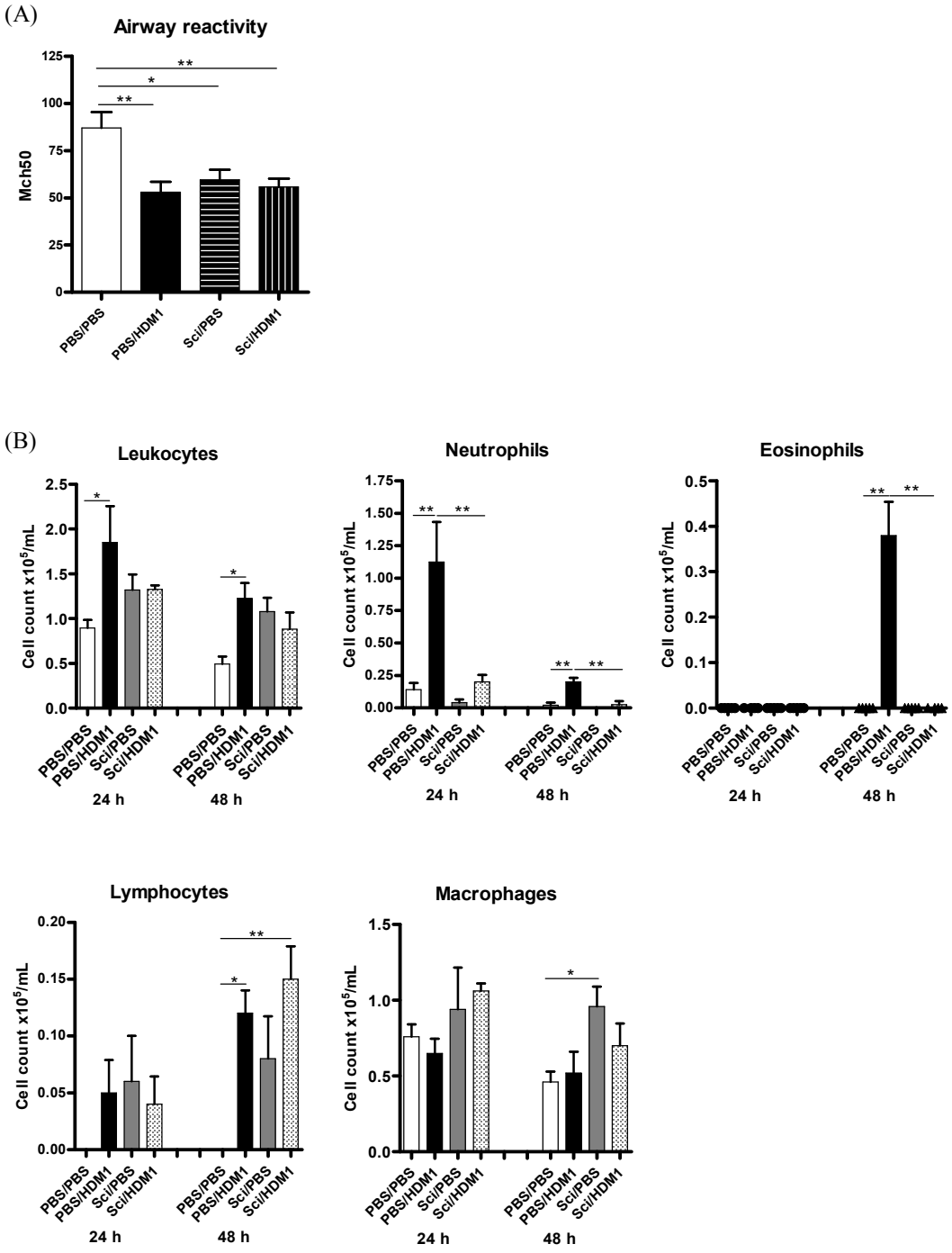


Figure 3.15 *S. sciuri* attenuated airway inflammation in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. (A) 12 hours after the last instillation, aiway reactivity was measured by Head-out body plethysmography. (B) 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively, and cell differentiation of leukocytes in the BAL fluid was performed. $^{*}p < 0.05$, $^{**}p < 0.01$

3.4.2 *S. sciuri* lowered levels of BAL cytokines and chemokines in HDM-induced murine asthma model

In addition to attenuating airway inflammation, pre-treatment of *S. sciuri* in HDM-mice prevented the secretion of CXCL1 and IL-5 into the BAL fluid at the first time point (24 hours after the last instillation). At the second time point, 48 hours after the last instillation, the level of CXCL1 in HDM-mice was still lowered by the bacteria, while the level of IL-5 in HDM-sensitized mice recovered to a normal level. The chemokine CCL11 (eotaxin) was not detectable at both time points.

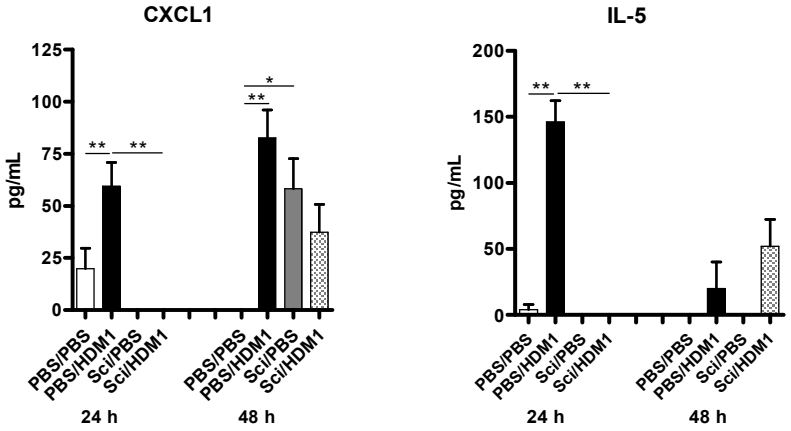


Figure 3.16 *S. sciuri* lowered protein levels of BAL CXCL1 and IL-5 in HDM-induced murine asthma mode

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. Protein levels of CXCL1 and IL-5 in the BAL fluid were determined by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$

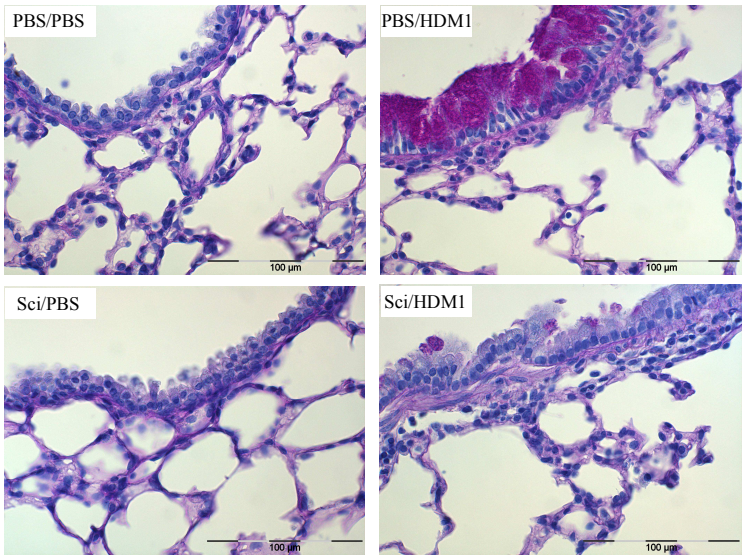
3.4.3 *S. sciuri* exerted no effect on the production of serum immunoglobulins in HDM-induced murine asthma model

No apparent effects on the titers of HDM-IgG1, HDM-IgG2a and total IgE were exerted in HDM-mice when pre-treated with *S. sciuri*.

3.4.4 *S. sciuri* alleviated goblet cell hyperplasia and mucus hypersecretion in HDM-induced murine asthma model

As depicted in Figure 3.17, pre-treatment of *S. sciuri* in HDM-sensitized mice reduced the percentage of goblet cells and the volume of mucus content. This histological change was observed at both time points.

(A)



(B)

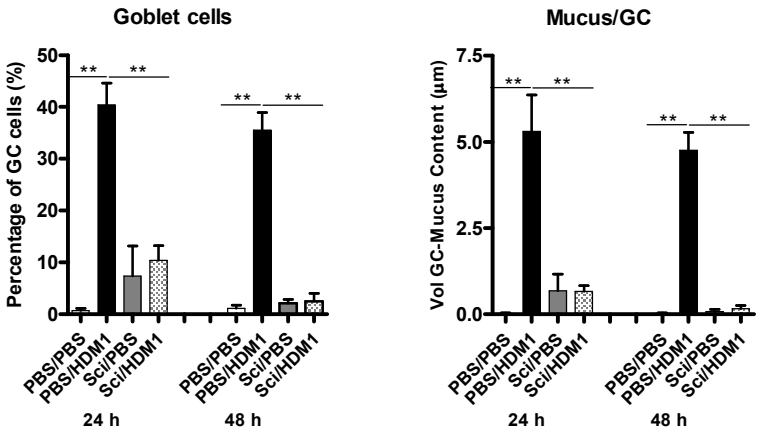


Figure 3.17 *S. sciuri* abolished goblet cell hyperplasia and mucus hypersecretion in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively, and the lungs were subjected to histological examinations. (A) Representative photomicrographs of PAS-stained lung sections. (B) Proportion of goblet cells and the volume of mucus content were determined by using the software CAST. $^{**}p < 0.01$

3.4.5 *S. sciuri* inhibited cytokine productions from *in vitro* restimulated lymphocytes in HDM-induced murine asthma model

Pre-treatment of *S. sciuri* had an inhibitory impact on cytokine production from HDM-restimulated lymphocytes at the first time point. As shown in Figure 3.18, the levels of IL-4, IL-5, IL-13, IFNγ, IL-10 and IL-17A were significantly lower in HDM-mice with *S. sciuri* treatment than without treatment, while the levels of IL-9 and GM-CSF were not significantly affected by *S. sciuri* treatment. At the second time point, the levels of the above detected cytokines in HDM-mice with or without bacterium treatment were comparable to those of PBS-mice.

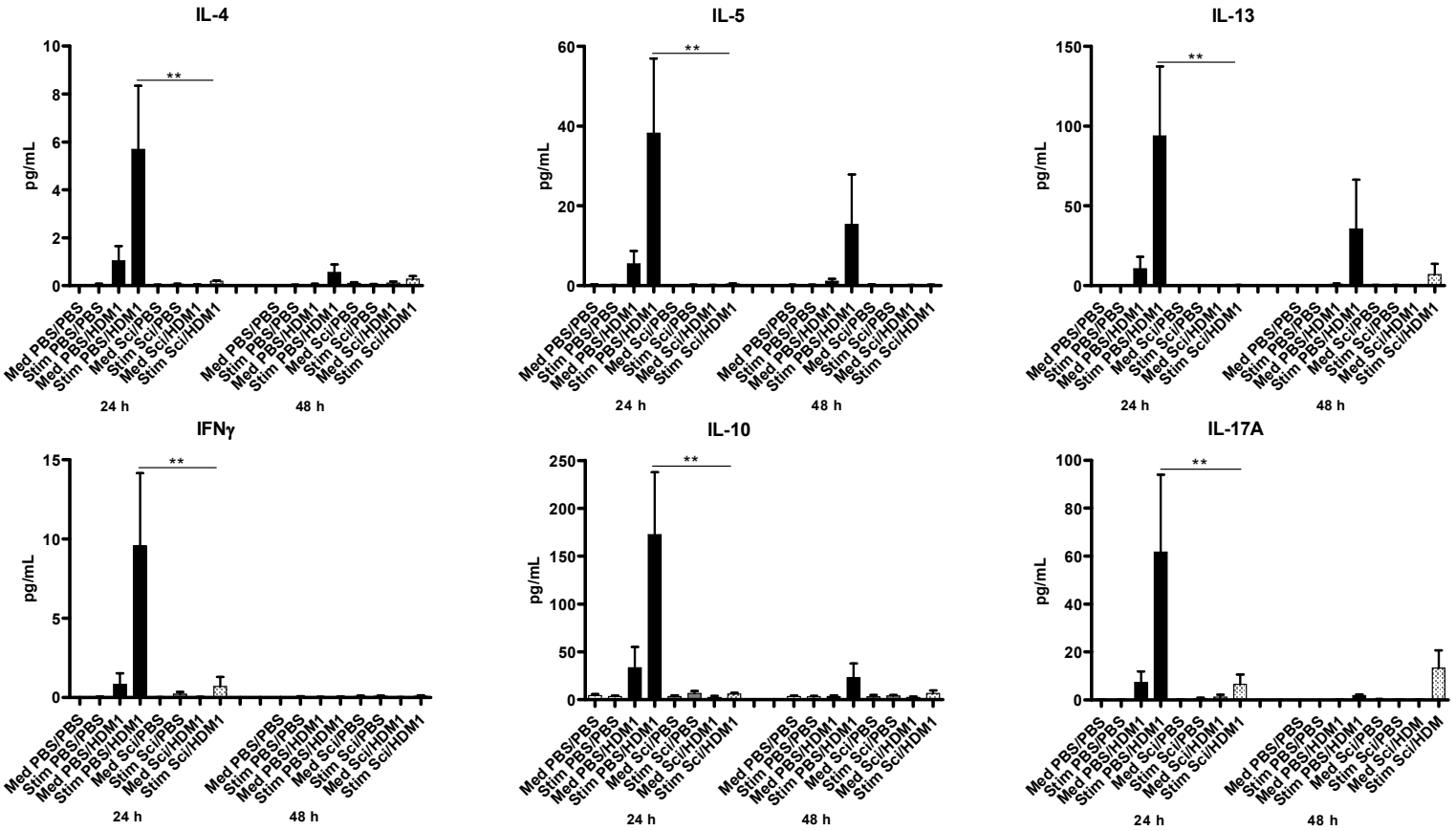


Figure 3.18 (to be continued) *S. sciur*i inhibited cytokine production from *in vitro* restimulated lymphocytes in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. Mediastinal lymphocytes were harvested and restimulated with 50 µg/ml HDM1 for 72 hours *in vitro*. The levels of cytokines IL-4, IL-5, IL-13, IFNγ, IL-10, IL-17A, IL-9 and GM-CSF in the cultured supernatant were determined by using CBA multiplex. To serve as negative control, lymphocytes were incubated with medium. $^{*}p < 0.05$, $^{**}p < 0.01$

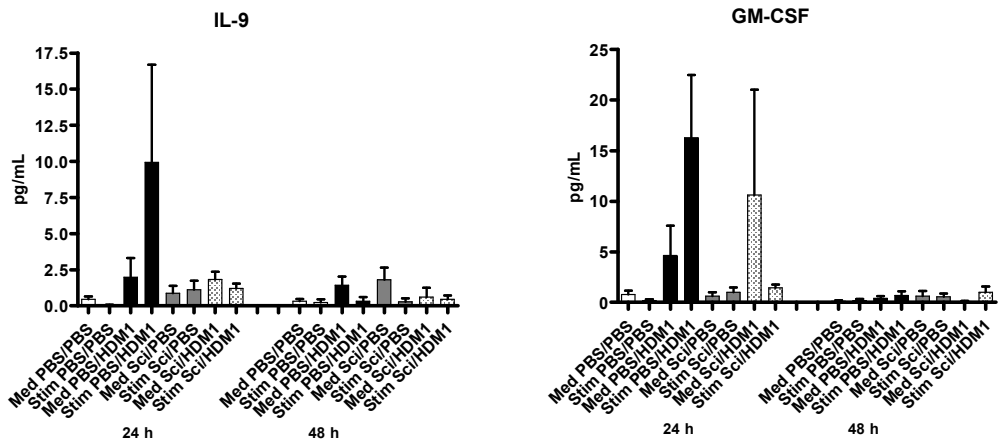


Figure 3.18 (continued) *S. sciuri* inhibited cytokine production from *in vitro* restimulated lymphocytes in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. Mediastinal lymphocytes were harvested and restimulated with 50 µg/ml HDM1 for 72 hours *in vitro*. The levels of cytokines IL-4, IL-5, IL-13, IFNγ, IL-10, IL-17A, IL-9 and GM-CSF in the cultured supernatant were determined by using CBA multiplex. To serve as negative control, lymphocytes were incubated with medium. $^{*}p < 0.05$, $^{**}p < 0.01$

3.4.6 *S. sciuri* repressed the mRNA expressions of asthma-related genes in HDM-induced murine asthma model

As shown in Figure 3.19, pre-treatment of *S. sciuri* abrogated the upregulation of mRNA expression of immune response genes CXCL1, CCL11 (eotaxin), CCL17, IL-4, IL-5 and IL-13 in HDM-sensitized mice.

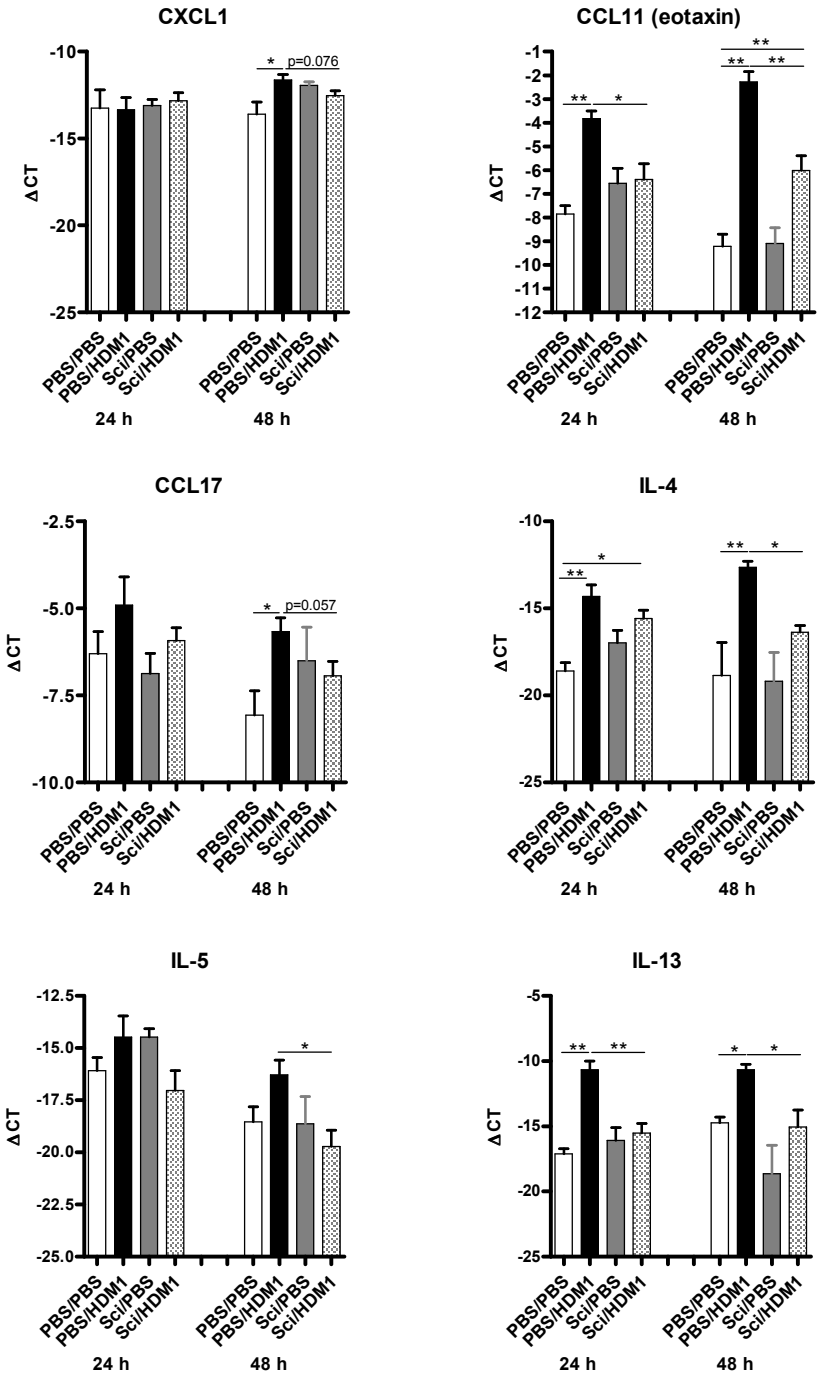


Figure 3.19 *S. sciuri* repressed the mRNA expressions of asthma-related genes in HDM-induced murine asthma model

BALB/c mice (n = 5 per group) were instilled intranasally with 100 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. The lungs were homogenized and mRNA expression of the asthma-related genes CXCL1, CCL11 (eotaxin), CCL17, IL-4, IL-5 and IL-13 were quantified by QPCR. $^{*}p < 0.05$, $^{**}p < 0.01$

3.5 The inhibitory role of *S. sciuri* in high dose HDM-induced murine asthma model

It was reported that higher dose of HDM extract induced a stronger asthma phenotype (Ward et al., 2011). But could *S. sciuri* still provide protection in such a model? In this experiment, 345 μg of the whole extract HDM1, which equals to 100 μg of the total protein, was used instead of 100 μg HDM1 to sensitize the mice. Administrations of *S. sciuri* and HDM1 were carried out according to sensitization protocol 3 (Figure 2.3). 24 hours and 48 hours after the last instillation of HDM1, the mice were sacrificed and asthma-related parameters were analyzed dynamically. The bacterial strain *S. sciuri* was isolated from the dust of a farm cowshed and HDM1 was provided by Allergopharma Co. (see Table 3.5). To exclude pathogenicity from *S. sciuri*, mice only treated with the bacterium served as negative control.

Table 3.5 A brief data sheet of HDM1

Name	Company	Batch No.	*Der p* 1 content	*Der p* 2 content	Protein content
HDM1	Allergopharma	W0657678	4.49 μg/100 μg	2.35 μg/100 μg	29 μg/100 μg

3.5.1 *S. sciuri* inhibited neutrophil but not eosinophil airway inflammation in high dose HDM-induced murine asthma model

It was observed that HDM1 at a dose of 345 μg still elicited a mixed inflammation of neutrophils and eosinophils. This airway inflammation was only partially inhibited by administration of *S. sciuri*. As shown in Figure 3.20, in mice sensitized by HDM1, a substantial number of neutrophils and eosinophils fluxed into the airway 24 hours after the last instillation of HDM1, while administration of *S. sciuri* prevented the influx of neutrophils, but not eosinophils. 48 hours after the last instillation of HDM1, neutrophils were still inhibited by the bacterial treatment.

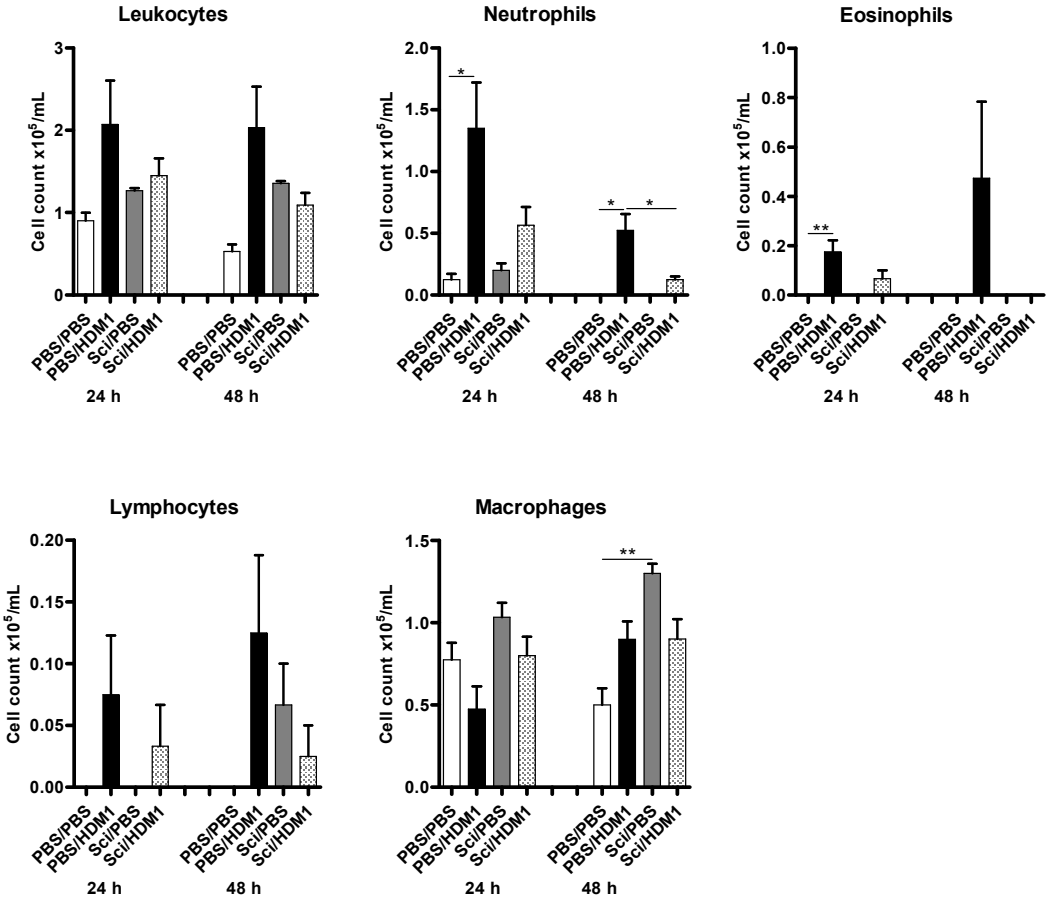


Figure 3.20 *S. sciuri* inhibited neutrophil but not eosinophil airway inflammation in high dose HDM-induced murine asthma model

BALB/c mice (n = 4 per group) were instilled intranasally with 345 μg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively, and cell differentiation of leukocytes in the BAL fluid was performed. $^{*}p < 0.05$, $^{**}p < 0.01$

3.5.2 *S. sciuri* prevented the secretion of BAL CXCL1, but not IL-5 in high dose HDM-induced murine asthma model

Sensitizing of HDM1 at a dose of 345 μg elicited a hypersecrection of BAL CXCL1 and IL-5 at the first time point. At the second time point, the level of CXCL1 remained higher in HDM-sensitized mice than in PBS-mice, while IL-5 returned to a normal level. This hypersecretion of CXCL1 in HDM-sensitized mice was prevented

by administration of *S. sciuri*, while IL-5 was not affected by the bacteria. The chemokine CCL11 (eotaxin) was undetectable in all groups (see Figure 3.21).

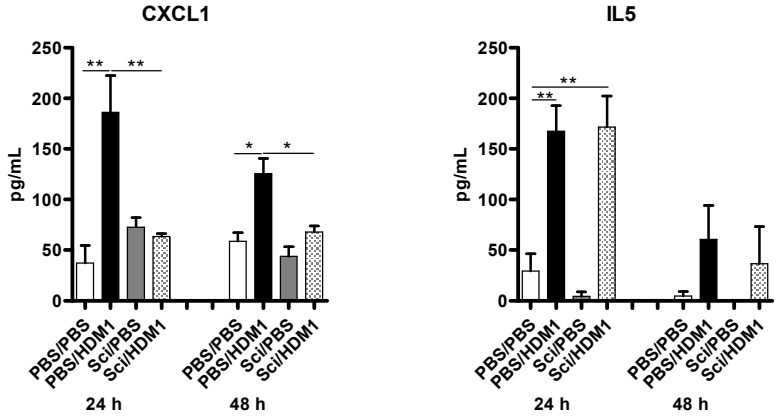


Figure 3.21 *S. sciuri* prevented the secretion of BAL CXCL1, but not IL-5 in high dose HDM-induced murine asthma model

BALB/c mice (n = 4 per group) were instilled intranasally with 345 μg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. Protein levels of CXCL1 and IL-5 in the BAL fluid were determined by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$

3.5.3 *S. sciuri* had no effect on the production of serum HDM-IgG1 in high dose HDM-induced murine asthma model

As shown in Figure 3.22, sensitization of the mice with 345 μg HDM1 led to a significant increase in serum HDM-IgG1 24 hours after the last instillation. At this time point, no HDM-IgG2a was detectable in HDM-sensitized mice and the level of total IgE remained comparable to that of PBS-mice. 48 hours after the last instillation, no significant difference in the titers of the the serum immunoglobulins (HDM-IgG1, HDM-IgG2a and total IgE) was found between the sensitized mice and non-sensitized mice. Pre-treatment of *S. sciuri* had no obvious effect on the production of HDM-IgG1 or HDM-IgG2a in HDM-sensitized mice. In contrast, the production of total IgE in HDM-mice was moderately lowered when treated with *S. sciuri*.

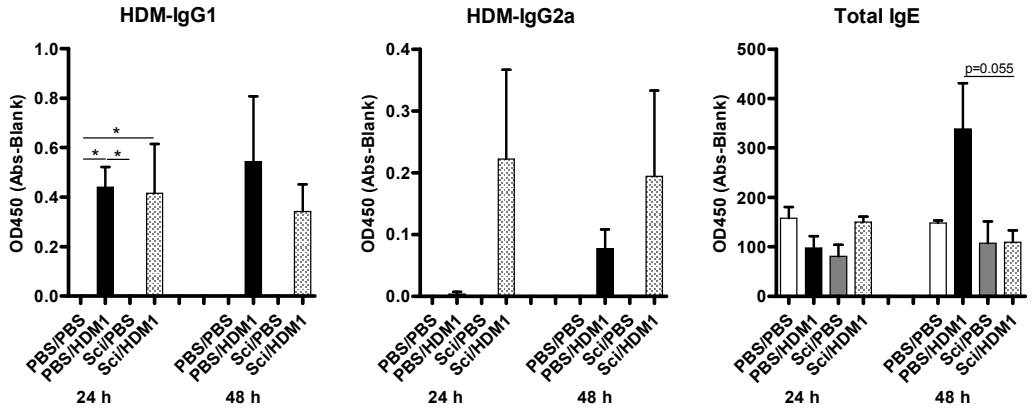


Figure 3.22 *S. sciuri* had no effect on serum HDM-IgG1 in high dose HDM-induced murine asthma model

BALB/c mice (n = 4 per group) were instilled intranasally with 345 μg HDM1 once a week for 4 times. Treatment of S. *sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. The titers of serum HDM-IgG1, HDM-IgG2a and total IgE were determined by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$

3.5.4 *S. sciuri* alleviated goblet cell hyperplasia and mucus hypersecretion in high dose HDM-induced murine asthma model

At both time points, goblet cell hyperplasia and mucus hypersecretion were observed in mice sensitized by 345 μg HDM1, which were reversed by administration of the bacterium *S. sciuri* (see Figure 3.23).

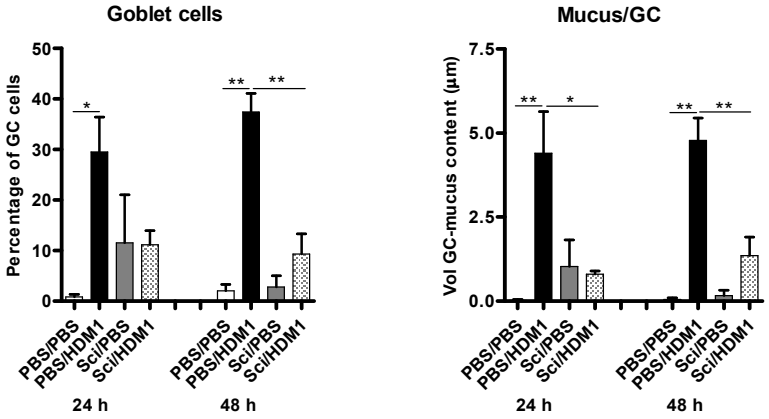


Figure 3.23 *S. sciuri* alleviated goblet cell hyperplasia and mucus hypersecretion in high dose HDM-induced murine asthma model

BALB/c mice (n = 4 per group) were instilled intranasally with 345 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively, and proportion of goblet cells and the volume of mucus content were determined by use of microscopic examination and analysis of the software CAST. $^{*}p < 0.05$, $^{**}p < 0.01$

3.5.5 *S. sciuri* diminished cytokine productions from *in vitro* restimulated lymphocytes in high dose HDM-induced murine asthma model

In mice sensitized by 345 µg HDM1, the restimulated lymphocytes showed increased activity with regards to cytokine production at the first time point. As shown in Figure 3.24, HDM-restimulated lymphocytes from the sensitized mice produced a higher level of IL-5, IL-13, IFNγ, IL-10 and IL-9 at the first time point, compared with the second time point. The levels of IL-4, TNFα and G-CSF were comparable between the two time points. The above mentioned cytokines were not inducible when the lymphocytes from PBS-mice were stimulated with HDM1 *in vitro*.

Admininstration of *S. sciuri* diminished the cytokine producing activity of lymphocytes in HDM-sensitized mice, in which the levels of IL-4, IL-5, IL-13, IFNγ, IL-10 and IL-9 were markedy reduced, while the level G-CSF remained unaffected by the bacterial treatment.

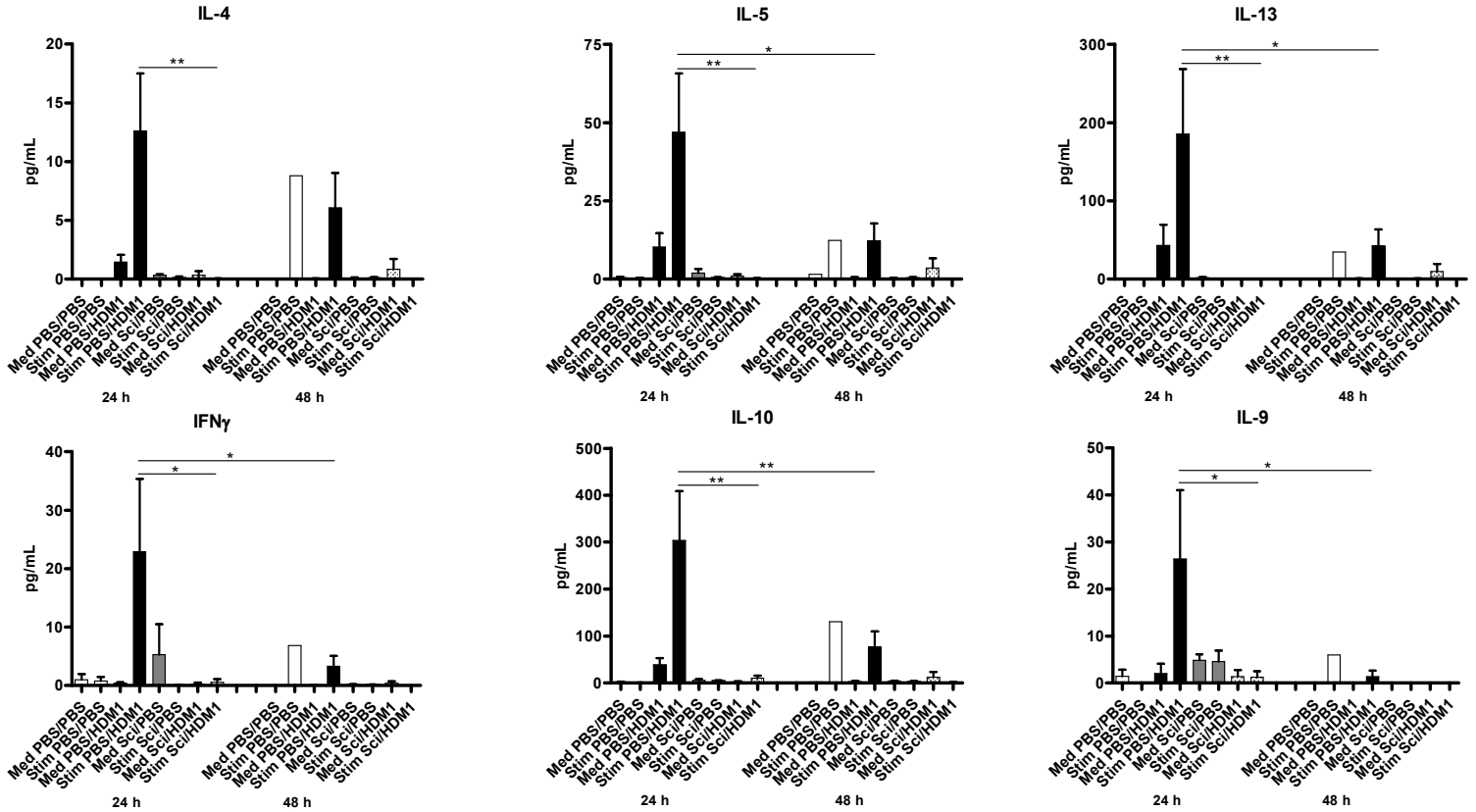


Figure 3.24 (to be continued) *S. sciuri* diminished cytokine production from *in vitro* restimulated lymphocytes in high dose HDM-induced murine asthma model

BALB/c mice (n = 4 per group) were instilled intranasally with 345 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. Mediastinal lymphocytes were harvested and restimulated with 172.5 µg/ml HDM1 for 72 hours *in vitro*. The levels of cytokines IL-4, IL-5, IL-13, IFNγ, IL-10, IL-9 and G-CSF in the cultured supernatant were determined by using CBA multiplex. To serve as a negative control, lymphocytes were incubated with medium. $^{*}p < 0.05$, $^{**}p < 0.01$

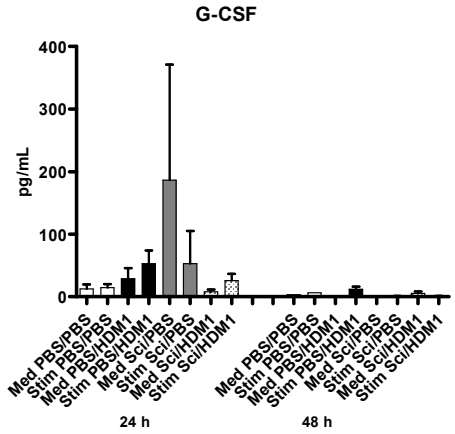


Figure 3.24 (continued) *S. sciuri* diminished cytokine production from *in vitro* restimulated lymphocytes in high dose HDM-induced murine asthma model

BALB/c mice (n = 4 per group) were instilled intranasally with 345 µg HDM1 once a week for 4 times. Treatment of *S. sciuri* was carried out nine days before the first instillation of HDM1, three times in one week, until the last instillation. 24 hours and 48 hours after the last instillation, the mice were sacrificed, respectively. Mediastinal lymphocytes were harvested and restimulated with 172.5 µg/ml HDM1 for 72 hours *in vitro*. The levels of cytokines IL-4, IL-5, IL-13, IFNγ, IL-10, IL-9 and G-CSF in the cultured supernatant were determined by using CBA multiplex. To serve as a negative control, lymphocytes were incubated with medium. $^{*}p < 0.05$, $^{**}p < 0.01$

To summarize, in the second part of this study, administration of the bacterium *S. sciuri* had an extensive impact on the reduction of HDM-induced murine asthma phenotype, which was demonstrated as follows: intranasal administration of *S. sciuri* before HDM sensitization alleviated both neutrophil and eosinophil airway inflammation, inhibited the secretion of CXCL1 and IL-5 into the BAL fluid and the production of cytokines IL-4, IL-5, IL-10, IL-13, IFNγ, IL-17A from the restimulated lymphocytes, and altered the histological manifestations by inhibiting goblet cell hyperplasia and mucus hyperproduction, whereas administration of *S. sciuri* in HDM-sensitized mice had no apparent effect on airway reactivity and the production of serum immunoglobulins. In mice sensitized by a higher dose of HDM1 (345μg), administration of *S. sciuri* only inhibited neutrophil airway inflammation, but not eosinophils.

Administration of *S. sciuri* alone without HDM sensitization did not lead to asthmatic manifestations in the mouse model, which was evidenced by the following: in mice which only received the bacteria, the counts of total leukocytes, neutrophils, eosinophils and lymphocytes remained comparable to that of PBS-mice, whereas the count of macrophages was significantly higher than that of PBS-mice at the second time point; the level of IL-5 and CCL11 were not detectable, whereas CXCL1 was found to be significantly higher at the second time point as compared to PBS-mice; the titers of serum immunoglobulins (HDM-IgG1, HDM-IgG2a, and total IgE) were comparable to that of PBS mice; no obvious production of the cytokines (IL-4, IL-5, IL-10, IL-13, IFNγ, IL-17A, IL-9, GM-CSF) were induced from the lymphocytes when stimulated with HDM1 *in vitro*; the percentage of goblet cells and the volume of mucus content remained comparable to those of PBS-mice. However, in mice which only received the bacteria, airway reactivity was significantly higher than that of PBS-mice.

4 Discussion

Allergic asthma is an inflammatory airway disease which develops based on gene-environment interactions. It is characterized by intense infiltration of airway inflammatory cells, increased airway responsiveness and reversible airway obstruction. This disease was initially thought to be Th2-dominated, in which eosinophils and lymphocytes were the predominating cells infiltrated in the airways and a limited set of cytokines including IL-4 and IL-5 were detected (Jeffery, 1992; Groot et al., 2002). Therefore, research into the underlying mechanisms of asthma pathogenesis was mainly focused on Th2 immune responses and the most frequently used model allergen OVA has gained great achievements in mimicking this subtype of asthma, namely eosinophil asthma phenotype or Th2-high asthma phenotype. Therapeutically, targeting eosinophil biology or Th2 cytokine such as IL-5 has been proved to be effective in asthma treatment (Wegmann, 2011; Sel et al., 2008; Wegmann et al., 2007; Molfino et al., 2011).

However, novel subtypes of asthma such as non-eosinophilic asthma phenotype has been identified and strategies of targeting eosinophils or Th2 cytokines may not be sufficient for the various phenotypes. The sensitizing allergen exploited in murine asthma models has shifted from the surrogate OVA to HDM extracts for the benefit of clinical relevance (Gregory et al., 2011). Recent studies demonstrated that HDM allergens not only induced an adaptive Th2 immune response, but also orchestrated innate immune pathways such as TLR4 or Toll/IL-1 signalling (Jacquet, 2011; Hammad et al., 2009; Phipps et al., 2009), in which airway neutrophilia is a recognised feature.

The Hygiene hypothesis has gained much attention and been frequently confirmed in the studies of allergic diseases. In OVA asthma models, some microbes including *Bordetella pertussis*, *Chlamydia*, *A. lwoffii F78* and *Lactococcus lactis* have been shown to counteract eosinophilic airway inflammation and provide protection against asthma. In the study of Ege and coworkers, the Gram-positive bacterium *S. sciuri* has been indicated as a protective factor in childhood asthma (Ege et al., 2011); yet this

asthma-protective effect has not been proven in a murine model so far. Therefore, the present study was designed to evaluate the potential effect of *S. sciuri* on HDM-induced murine asthma phenotype with a mixed neutrophil and eosinophil airway inflammation.

4.1 Allergic airway inflammation was elicited by intranasal sensitization of HDM extract

Current knowledge emphasizes that asthma development is a complex process resulting from interactions between genetic predisposition and environmental exposure (Renz et al., 2011; Mukherjee et al., 2011). Just like other allergic diseases, asthma is hereditary (Holloway et al., 2010). Children from asthmatic families are thought to be predisposed to asthma. Many powerful genetic tools were used to investigate the heritable component of asthma and recent genome-wide association studies (GWASs) identified more than 100 genes in 22 chromosomes as susceptibility factors for allergic asthma (Malerba et al., 2005; Vercelli, 2008). The list of asthma candidate genes is likely to expand in the coming years. However, most of the genes have only mild effects. Instead, environmental milieu was supposed to have a determinant function. It is generally agreed that multiple genes influence disease phenotypes through their interactions with the environment (Kleeberger et al., 2005) and even without any apparent genetic predisposition some individuals can develop asthma after exposure to environmental factors. Understanding the precise role of environmental exposure in the development of asthma is critical for understanding this disease (Schwartz, 2009). In contrast to genetic studies, research into the environmental field is still at an early stage. Despite the constantly increasing evidence for gene-environment effects in asthma pathogenesis, it is still obscure which environmental factors might be linked to a specific gene set (Kleeberger et al., 2005). Investigation of environmental effects in human subjects is still constrained due to variations across populations and the presence of ethical and technical issues. Compared with humans, mouse models are easily reproducible with a fixed genetic background and genetically more tractable, in which a strict genetic or environmental control could be achieved. Meanwhile, mouse models provide a fast and economic way to assess gene-environment relationship.

With the recognition of environmental effects on disease progression, it is now realized that some environmental factors might trigger disease onset, while other factors may confer disease protection. The double effects were observed in our models as well, which would be discussed separately. This part was mainly focused on the sensitizing effect of HDM extracts. In our models, HDM extract as an environmental allergen produced a profound effect on the development of an asthma phenotype. The three different origins of HDM extracts, which varied in their contents such as *Der p* 1 concentration, displayed unequal performance in eliciting airway inflammation. Among them, HDM1 (from Allergopharma Co.) induced the most robust influx of both neutrophils and eosinophils into the airways, HDM2 (from Greer Labs) exerted stronger effect on the induction of neutrophil airway inflammation, while HDM3 (from Greer Labs) only elicited a very weak inflammatory response. The divergent inflammatory responses induced by the three different extracts could be attributed to their diversity in relative composition and extract methods. In line with this, Post (Post et al., 2011) demonstrated that the composition of house dust mite extract has a crucial effect on epithelial remodeling and airway inflammation. Besides composition, different doses of HDM extracts had different inflammation-promoting abilities. The sensitizing dose could influence the aggregation of inflammatory cells and the production of BAL cytokines and chemokines and eventually affect asthma pathogenesis (Kurup et al., 2001). In regard to this, Delayre-Orthez proved that effects of endotoxins on allergen sensitization and challenge were dose-dependent (Delayre-Orthez et al., 2004). And in the study of Ward and coworkers (Ward et al., 2011), HDM-challenged mice displayed a dose-dependent increase in neutrophil, eosinophil and lymphocyte counts. Consistent with the data, our results showed that in mice sensitized by 10 μg HDM, no obvious asthmatic response was observed, while 100 μg HDM extract elicited a more distinct asthma phenotype than 50 μg HDM extract. It was proposed that even a higher dose of HDM extract (345 μg) would have a stronger effect than 100 μg, although no direct comparison was performed between the two doses. Just like with the other allergens, severity of the HDM effect could also rely on the sensitizing time. Due to different protocols employed in different studies, the optimum time for HDM sensitization is still

in debate. HDM extract was initially adopted by Cates et al. (Cates et al., 2004) to model acute allergic airway inflammation via the respiratory mucosa. They demonstrated that intranasal administration of HDM extract for 10 days without adjuvant provoked a Th2-type sensitization. Following this, numerous data of HDM models have been reported, but with wide variations. Our data revealed that intranasal instillation of HDM for 4 times in three weeks elicited a distinct asthma phenotype in the mouse model.

The pronounced effect of HDM extract on the induction of an asthma phenotype could be demonstrated by several biological observations in the present study. First of all, HDM sensitization resulted in the most unique feature of airway inflammation, which was dominated by neutrophils and eosinophils. As an early event, a large amount of neutrophils migrated into the airways 24 hours after the last instillation of HDM extract. This migration was possibly associated with the activations of cytokines and chemokines including CXCL1, IL-17 and IFNγ. CXCL1 has been identified as a potent neutrophil chemotactic factor both *in vivo* and *in vitro* (Bozic et al., 1995). Post and coworkers (Post et al., 2011) demonstrated that in HDM-treated mice, increased levels of CXCL1 were accompanied by increased numbers of neutrophils. This study revealed that in HDM-sensitized mice, both mRNA and protein expressions of CXCL1 were upregulated. Similar to CXCL1, IL-17 was induced by HDM sensitization at both mRNA and protein levels. IL-17 is a proinflammatory factor, which is known to induce productions of other cytokines and chemokines, especially CXCL1 (the human homologue of IL-8) (Breslow et al., 2011). IL-17 has been associated with severe asthma, in which much greater airway neutrophilia was recognized (Lajoie et al., 2010; Shannon et al., 2008). It was reported that increased levels of IL-17 could facilitate neutrophil infiltration (Witowski et al., 2004), and the production of IL-17 could be induced by rIL23 or HDM extract (Kohyama et al., 2007; Wu et al., 2007; Post et al., 2011). A third inflammatory factor increased by HDM sensitization is IFNγ. The interactions of IFNγ and neutrophils have been underestimated (Ellis et al., 2004). In the present study, the increased production of IFNγ from restimulated lymphocytes was

possible related to neutrophil airway inflammation. In support of this, Yoshimura (Yoshimura et al., 2007) demonstrated that IFN-γ effectively prolonged the survival of activated neutrophils. Additionally, HDM sensitization increased the mRNA and protein expressions of IL-10, an anti-inflammatory cytokine studied by numerous investigations. Due to controversial results obtained from different studies (Finkelman et al., 2010; Bettiol et al., 2002; Fu et al., 2006; Oh et al., 2002; Presser et al., 2008), the precise role of IL-10 in asthma pathogenesis is still not determined.

In comparison with neutrophils, recruitment of airway eosinophils occurred at a later time point after HDM sensitization. The activation of eosinophils was possibly related to upregulation of Th2 cytokines (IL-4, IL-5 and IL-13) and the inflammatory factor GM-CSF. Firstly, the elevation of IL-5 in mRNA and protein levels might contribute directly to the airway eosinophilia (Finkelman et al., 2010). Among the three classic Th2 cytokines (IL-4, IL-5 and IL-13), IL-5 is the best characterized for influencing eosinophil biology through the binding of IL-5R on eosinophil surface, and in humans IL-5 acts mainly on eosinophils and their precursors. It was reported that in murine asthma models overexpression of IL-5 in the lung led to spontaneous development of eosinophilic airway inflammation and IL-5 deficiency abolished this eosinophilia (Lee et al., 1997; Foster et al., 2012). It was also reported that anti-IL-5 mAb treatment in clinical asthma patients caused a considerable decrease in blood eosinophilia and a partial decrease in lung eosinophilia (Leckie et al., 2000; Flood-Page et al., 2003). In the present study, IL-5 protein levels were found to be increased in the BAL. In the *in vitro* restimulated lymphocytes the increase of IL-5 was accompanied by the increase of IL-4 and IL-13, two other cytokines which might exert indirect eosinophil-promoting effect. One feasible explanation for IL-4 and IL-13 inducing eosinophil activation is that both IL-4 and IL-13 signal through the type II IL-4R to induce production of chemokines, including eotaxin-1 (CCL11) and eotaxin-2 (CCL24), which attract eosinophils to the lungs (Finkelman et al., 2010; Munitz et al., 2008). Compared with IL-5, IL-4 and IL-13 worked more broadly in multiple pathways in the pathogenesis of asthma, which will be discussed later. In addition to the three Th2

cytokines, HDM sensitization increased the production of GM-CSF, a cytokine which was also implicated in eosinophil trafficking (Pope et al., 2001). As GM-CSF shares a common β chain receptor, it was deduced that GM-CSF and IL-5 may share some signaling pathway which regulated esinophil differentiation, proliferation and survival (Molfino et al., 2011).

Although neutrophils and eosinophils constituted the major part of airway inflammation elicited by HDM extract, epithelial cells may play a critical part in the initiation of this inflammation. It is generally thought that the respiratory epithelium senses the signals produced by constant contacts of allergens and develops active responses (Holgate, 2011). The airway epithelium is a well-established sources of cytokines and chemokines (Müller et al., 2005). *In vitro* studies have demonstrated that stimulation of respiratory epithelium by HDM extract led to secretion of different chemokines such as CCL20 (Nathan et al., 2009). Our results indicated HDM sensitization activated epithelial cells possibly through the upregulation of chemokine genes CXCL1, CCL11 (eotaxin) and CCL17. The three genes share the same cellular source, epithelial cells, but exerted different chemotactic capabilities. Among them, CXCL1 was expected to be responsible for neutrophil migration, CCL11 might be associated with eosinophil recruitment (Finkelman et al., 2010), and CCL17 was probably related to the chemotaxis of Th2 cells (Yao et al., 2011). Based on the above data, it was proposed that both innate and adaptive immune mechanisms were involved in the development of allergic airway inflammation in HDM-sensitized together with a strong involvement of epithelial cell activation.

In addition to airway inflammation, further features of an asthmatic response were also induced by HDM exposure including overproduction of HDM-specific IgG1 and goblet cell hyperplasia, which have been frequently indicated in the studies of allergic airway diseases (Kaneko et al., 1995; Ishikawa et al., 2011; Wegmann et al., 2005; Vermeer et al., 2003). The structural changes of the airways such as goblet cell hyperplasia and mucus hypersecrection were probably correlated with the elevation of IL-13 (Tanabe et al., 2011)(Zhu et al., 1999). Also, it should be noted that the airway

reactivity in HDM-sensitized mice did not correspond to the development of airway inflammation. Although the dissociation of airway hyperresponsiveness from airway inflammation was not uncommon, a definite explanation is still lacking. It was argued that AHR was influenced by the genetic background of the mouse, the method used, the time point of analysis, etc. In our case, airway reactivity was measured by Head-out body plethysmography, a non-invasive method, which may not directly correlate with changes in airway resistance (Bates et al., 2004). Therefore, it would be recommended to confirm these results by an invasive measurement for airway reactivity.

Collectively, our results demonstrated that HDM extract was a critical determinant for the development of a distinct murine asthma phenotype, which was characterized by a unique airway inflammation of neutrophils and eosinophils, an augmented IgG1 response and subsequent histological changes. The pathogenesis of this phenotype was a complex process, with multiple components involved in. The unique gene expression pattern further suggested that the initiation of the unique airway inflammation was possibly due to a specific activation of airway epithelial cells.

4.2 Administration of *S. sciuri* as a possible preventive strategy for HDM-induced asthma phenotype

Environmental exposure has been proven to have a significant impact on the progression of allergic disease including asthma (Wahn, 2011). On one hand, exposure to environmental factors such as house dust mites, cockroaches and cat dander was considered as a dominant risk factor for the development or exacerbation of asthma in sensitized individuals (Sporik et al., 1999; Squillace et al., 1997; Litonjua et al., 2001; Polk et al., 2004). On the other hand, exposure to microbial compounds was found to influence the development of adaptive immunity and the resulting allergy possibly through the modulation of innate immune responses (Gregory et al., 2011; Braun-Fahrländer et al, 2002). The hygiene hypothesis predicts that infections may protect against asthma, which has been addressed by numerous studies. In Celedón's survey, it was concluded that early endotoxin exposure may be a protective factor

against atopy (Celedón et al., 2007). Epidemiological studies performed in Europe have frequently demonstrated that children raised on farms had a lower prevalence of asthma than those who lived in cities (Von Ehrenstein et al, 2000; Barnes et al., 2001; Riedler et al., 2000; Riedler et al., 2001; Ege et al., 2011). The most striking finding was that specific environmental exposure correlated with increased expression of specific genes (Ege et al., 2007; Vercelli, 2008). For example, haying was correlated with increased expression of TLR7 and TLR10, whereas feeding silage was related to higher expression of TLR6 and TLR8. Actually, not all farming environments protect against the development of asthma in children (Ege et al., 2007). Mouse models have been used to provide a proof-of-concept for the protective effects of farm-derived microbes. *Acinetobacter lwoffii* and *Lactococcus lactis* were the earliest studied cowshed bacteria, which have shown to exert asthma protective effects in mouse models (Debarry et al., 2007; Conrad et al., 2009; Brand et al., 2011). Meanwhile, other microbes including *Bacillus licheniformis* (Vogel et al., 2008), *Bifidobacterium longum* AH1206 (Lyons et al., 2010), *Helicobacter pylori* (Arnold et al., 2011), *Escherichia coli* (Nembrini et al., 2011), and *Broncho-Vaxom* (Navarro et al., 2011) have also been shown to modulate allergic airway reactions or provide asthma protection.

Our mouse model provided the first evidence that the potential asthma-preventive effect of *S. sciuri* was tested in a mouse model with a mixed eosinophil and neutrophil asthma phenotype, since this bacterium has only been indicated of a protective role in childhood asthma (Ege et al., 2011). In our model, intranasal administration of *S. sciuri* before HDM sensitization alleviated the mixed airway inflammation of neutrophils and eosinophils, in which the decrease of eosinophils was not accompanied by a tendency toward higher neutrophil counts, or vice verse. Additionally, administration of *S. sciuri* in HDM-sensitized mice abrogated the secretion of CXCL1 and IL-5 into the BAL and the production of cytokines IL-4, IL-5, IL-10, IL-13, IFNγ, IL-17A from the restimulated lymphocytes was also reduced. Histologically, pre-treatment of *S. sciuri* altered the asthmatic manifestations by inhibiting goblet cell hyperplasia and mucus hyperproduction. However, administration of *S. sciuri* in HDM-sensitized mice had no

apparent effect on airway reactivity and the production of serum immunoglobulins.

There is accumulating data that microbes could confer allergy protection, yet the underlying mechanisms are still conflicting. Previous studies were conducted mostly in OVA asthma models, in which eosinophils were the predominant inflammatory cells and administration of microbes led to the inhibition of eosinophilc airway inflammation. In Debarry's study (Debarry et al., 2007), it was proposed that the bacteria conducted protective effect against allergy possibly through promoting Th1 and inhibiting Th2 activity. Lyons and colleagues (Lyons et al., 2010) attributed the protective role of bacteria to the airway recruitment of regulatory T cells. Brand (Brand et al., 2011) recognized that microbes provide asthma protection through epigenetic regulation in a prenatal window of opportunity. In the present study, both eosinophils and neutrophils constituted the major part of airway inflammation induced by HDM extract. Administration of *S. sciuri* in HDM-mice abrogated the increased mRNA expressions of a variety of genes CXCL1, CCL11 (eotaxin), CCL17, IL-4, IL-5 and IL-13, which was corresponded by the decreased protein levels of CXCL1, IL-4, IL-5 and IL-13. Moreover, protein levels of the cytokines IFNγ and IL-10 were also lowered by the bacterium. The distinct gene expression pattern, together with the reduced protein productions observed in *S. sciuri*-treated HDM-mice suggested the protective effect of *S. sciuri* might not be mediated through inducing Th1 or regulatory T cells. An alternative proposal was that this bacterium might provide asthma protection through the modulation of immune responses, since epithelilal cells are constanly receiving the bacterial contact and might act as a master regulator of both innate and adaptive immune responses (Bulek et al., 2010).

Interestingly, this protective role of *S. sciuri* became weaker when the mice were sensitized by 345 μg HDM1. In mice sensitized by this dose of HDM1, administration of *S. sciuri* only inhibited neutrophil airway inflammation, but not eosinophils, indicating that neutrophils were more sensitive to the protective effects of *S. sciuri* than eosinophils. Administration of *S. sciuri* still alleviated goblet cell hyperplasia and mucus hypersecretion in mice sensitized by a higher dose of HDM1.

Taken together, data obtained from the present study provided the first evidence that the bacterium *S. sciuri* had an extensive effect on the prevention of a mixed murine asthma phenotype. Administration of *S. sciuri* in HDM-sensitized mice led to the inhibition of the unique airway inflammation, goblet cell hyperplasia and mucus hypersecretion, accompanied by a down regulation of various chemokines and cytokines including CXCL1, IL-5, IL-4, IL-10, IL-13, IFNγ and IL-17A.

These data further supported the concept of hygiene hypothesis in a HDM murine asthma model. The possible mechanism by which *S. sciuri* conducted its protective effect might be through the regulation of mRNA expression of immune response genes including CXCL1, CCL11 (eotaxin), CCL17, IL-4, IL-5 and IL-13, which was distinct from the previous studies. Due to the few number of genes examined, understanding of this mechanism is still limited. It's tempting to fully disclose the relationship between *S. sciuri* and asthma and translate the findings into patient benefit.

Therefore, it was concluded that intranasal administrations of *S. sciuri* might serve as a promising strategy for asthma prevention.

5 Summary

5.1 Summary

Allergic asthma is a chronic inflammatory disease of the airways based on a dysregulated immune response to innocuous antigens (allergens) with a predominance of Th2-driven activities. According to the hygiene hypothesis, neonatal and early childhood exposure to certain microbes and their products may protect them from the development of allergic responses due to a shift in the balance of T cell subpopulation activities. This concept has been experimentally proven for Gram-negative bacteria in a mouse model representing an eosinophil granulocyte-dominated phenotype using ovalbumin as model allergen. Recently, epidemiological studies revealed a specific association between *Staphylococcus sciuri* exposure and reduced asthma prevalences.

The aim in the first part of this work was to establish a murine model of allergic airway inflammation with a more pronounced contribution of neutrophil granulocytes, using the clinically relevant house dust mite (HDM) allergen. Various asthma-relevant parameters were monitored after exposure to house dust mite extracts from different sources, at a wide range of doses and at various intervals after allergen challenge. It could be revealed that HDM1 from Allergopharma Co. (Hamburg, Germany) elicited the strongest asthma phenotype with a mixed eosinophil and neutrophil airway inflammation, an increased production of serum HDM-IgG1 and subsequent histological changes. This phenotype was also more pronounced in mice sensitized with the highest tested HDM extract concentration (100 µg). It also became evident that neutrophils and eosinophils got involved at different time points of the inflammatory process by the action of different chemotactic factors and their activation thus representing different biological events.

In the second part of this work, the Gram-positive bacterium *S. sciuri* was tested for its potential allergy-protective effect in this model. Exposure to *S. sciuri* strongly inhibited the generation of HDM extract-induced airway inflammation, as evidenced by a reduction of both eosinophil and neutrophil numbers compared to HDM

extract-induced airway inflammation in non-exposed control-animals. This suppression of airway inflammation was accompanied by diminished production of cytokines and chemokines and by an alleviation of goblet cell hyperplasia and mucus production.

These data provide the first experimental evidence that exposure to *S. sciuri* might be protective against the development of allergic airway inflammation and associated pulmonary pathology.

5.2 Zusammenfassung

Das allergische Asthma ist eine chronisch entzündliche Erkrankung der Atmenwege, die auf einer dysregulierten Immunantwort gegenüber harmlosen Antigenen (Allergenen) mit einer dominanten Th2-Aktivität beruht. Gemäß der Hygienehypothese sind Neugeborene und Kinder, die in den ersten Lebensjahren bestimmten Bakterien und ihren Produkten ausgesetzt sind, vor der Entwicklung von allergischen Reaktionen geschützt. Dies beruht auf einer Verschiebung in der Balance der Aktivitäten verschiedener T-Zell-Subpopulationen. Experimentell konnte dieses Konzept bereits für Gram-negative Bakterien in einem Maus-Modell mit Ovalbumin als Allergen, welches einen von eosinophilen Granulozyten dominierten Phänotyp aufweist, aufgezeigt werden. Kürzlich konnte in epidemiologischen Studien eine spezifische Assoziation zwischen einer *Staphylococcus sciuri* Exposition und einer verminderten Asthma Prävalenz aufgezeigt werden.

Das Ziel des ersten Teils der Arbeit war es, ein Maus-Modell der allergischen Atemwegsentzündung mit einer verstärkten Beteiligung von neutrophilen Granulozyten zu entwickeln. Hierfür wurde als klinisch relevantes Allergen Hausstaubmilben(HDM)-Extrakt verwendet. Es wurde der Einfluss von HDM-Extrakten verschiedener Quellen, unterschiedlicher Dosen, verschiedene Zeitintervalle nach letzter Allergengabe auf Asthma-relevante Parameter untersucht. Hierbei konnte gezeigt werden, dass HDM1 von Allergopharma CO. (Hamburg, Germany) den stärksten Asthmaphänotyp mit einer gemischten Eosinophilen und Neutrophilen Atemwegsentzündung, einer erhöhten HDM-IgG1-Produktion und

entsprechenden histologischen Veränderungen induzierte. Dieser Phänotyp war zudem am stärksten in Mäusen ausgeprägt, die mit der höchsten getesteten HDM-Extrakt Dosis (100 µg) exponiert wurden. Es konnte außerdem gezeigt werden, dass Neutrophile und Eosinophile aufgrund verschiedener chemotaktischer Faktoren und deren Aktivierung zu verschiedenen Zeitpunkten des inflammatorischen Prozesses beteiligt sind und ihre Aktivierung somit differentielle biologische Ereignisse darstellt.

Im zweiten Teil dieser Arbeit wurde das Gram-positive Bakterium *S. sciuri* hinsichtlich potentieller allergieprotektiver Eigenschaften in diesem Modell getestet. Exposition des Bakteriums *S. sciuri* führte zu einer sehr ausgeprägten Verminderung der HDM-induzierten Atemwegsentzündung. Dies spiegelte sich in einer Verminderung sowohl der Zahl eosinophiler als auch neutrophiler Granulozyten, im Vergleich zu den nicht *S. sciuri*-exponierten Kontrolltieren wider. Parallel zur reduzierten Atemwegsentzündung wurde eine verminderte Produktion von Zytokinen und Chemokinen und eine Abschwächung der Goblet-Zell-Hyperplasie und Mukus-Produktion beobachtet.

Diese Daten geben erste experimentelle Hinweise darauf, dass die Exposition mit *S. sciuri* eine protektive Wirkung auf die Entwicklung einer allergischen Atemwegsentzündung und damit assoziierter pulmonärer Pathology haben kann.

6 Reference

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7 List of abbreviations

AHR	aAirway hyperresponsiveness
A. lwoffii F78	*Acinetobacter lwoffii* F78
APCs	antigen-presenting cells
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CBA	cytometric bead array
cDNA	complementary DNA
DCs	dendritic cells
Der f	*Dermatophagoides farinae*
Der p	*Dermatophagoides pteronyssinus*
DNase I	deoxyribonuclease I
dsDNA	double-stranded DNA
EAR	early asthmatic reaction
ECM	extracellular matrix proteins
E. coli	*Escherichia coli*
EF	expiratory airflow
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
GATA-3	GATA binding protein 3
GCH	goblet cell hyperplasia
GINA	Global Initiative for Asthma
GRO-α	growth-related oncogene α
GWASs	genome-wide association studies
HDM	house dust mite
HDM1 (2,3)	house dust mite extract 1 (2,3)
HDM-IgG1 (IgG2a, IgE)	HDM-specific IgG1 (IgG2a, IgE)
HRP	horseradish peroxidase
IFNγ	interferon γ
i.n.	intranasally
i.p.	intraperitoneally
IgE	immunglobulin type E
IgG1	immunglobulin type G1

IgG2a	iImmunglobulin type G2a
KC	keratinocyte chemokine
L(*P*)	the length per test point
LAR	Late asthmatic reaction
MAPK	Mitogen-activated protein kinase
MBP	major basic protein
MCh	methacholine
min	minute
MIP2	macrophage inflammatory protein 2
N_{EC}	the number of epithelial cells
N_{GC}	the number of goblet cells
NLRs	Nod-like receptors
OVA	ovalbumin
PAS	Periodic acid-Schiff
PBS	phosphate buffered saline
PE	phycoerythrin
PCR	polymerase chain reaction
POD	Peroxidase
PRRs	pattern recognition receptors
QPCR	quantitative real time polymerase chain reaction
SEM	standard error
S. sciuri	*Staphylococcus sciuri*
Th2 cell	T helper 2 cell
TLRs	Toll-like receptors
Tm	melting temperature
TNFα	tumor necrosis factor alpha
Treg cell	regulatory T cells
TSLP	thymic stromal lymphopoietin
V_{Mucus}	the volume of GC-mucus content
WT	wild type

8 List of Figures and Tables

8.1 Figures

8.2 Tables

9 List of academic teachers

My academic teachers are the professors from Marburg, Germany including Blaser, Gemsa, Renz, Garn, Pfefferle.

The professors from China include Chen, Li, Lu, Ma, Shi, Wan, Wang, Yang, Yu, Zhang, Zhou.

10 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Development of a house dust mite model of mixed allergic airway inflammation and analysis of allergyprotective effects of *Staphylococcus sciuri*“ im Institut für Laboratoriumsmedizin und Pathobiochemie, Molekulare Diagnostik der Philipps-Universität Marburg unter Leitung von Prof. Renz ohne sonstige Hilfe selbst durchgefuehrt habe. Bei der Abfassung der Arbeit habe ich keine anderen als die in der Dissertation angeführten Quellen und Hilfsmittel benutzt und vollständig oder sinngemaess uebernommene Zitate entsprechend gekennzeichnet. Ich habe bisher weder an einem in oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch eine andere Arbeit als Dissertation oder die vorliegende zu anderen Prüfungszwecken vorgelegt.

Marburg, 29.03.2012

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(Min Zhao)

11 Curriculum Vitae

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12 Acknowledgement

The river of time flows rapidly, and takes me where I am now. Today, it's a special occasion for me. It will be a summary for my last 1255 days when I study for the doctor degree. It will also be an introduction to my future days.

For me, the dissertation is like my child. From pregnance, to development, to maturity, and now it comes out. Sometimes it went hardly, and I tried to overcome it. Finally I managed to get through it. I'm happy about it, for I grow up together with the child. And I come to understand a famous Chinese saying: happiness lies in the process when you persuit it, but not in the result you have got. I think the same principle works with my dissertation. For the completion of it, I'd like to extend my thanks to all the people who help me. Without their dedication and hard work, this dissertation would not have been possible.

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The river of time flows rapidly, and pushes me forward to what I wish to be.